



Modulation of the Interaction between Neurotensin Receptor NTS1 and Gq Protein by Lipid

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Membrane lipids have been implicated to influence the activity of G-protein-coupled receptors (GPCRs). Almost all of our knowledge on the role of lipids on GPCR and G protein function comes from work on the visual pigment rhodopsin and its G protein transducin, which reside in a highly specialized membrane environment. Thus, insight gained from rhodopsin signaling may not be simply translated to other nonvisual GPCRs. Here, we investigated the effect of lipid head group charges on the signal transduction properties of the class A GPCR neurotensin (NT) receptor 1 (NTS1) under defined experimental conditions, using self-assembled phospholipid nanodiscs prepared with the zwitter-ionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), the negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), or a POPC/POPG mixture. A combination of dynamic light scattering and sedimentation velocity showed that NTS1 was monomeric in POPC-, POPC/POPG-, and POPG-nanodiscs. Binding of the agonist NT to NTS1 occurred with similar affinities and was essentially unaffected by the phospholipid composition. In contrast, Gq protein coupling to NTS1 in various lipid nanodiscs was significantly different, and the apparent affinity of Gαq and Gβ₁γ₁ to activated NTS1 increased with increasing POPG content. NTS1-catalyzed GDP/GTPγS nucleotide exchange at Gαq in the presence of Gβ₁γ₁ and NT was crucially affected by the lipid type, with

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Abbreviations used: [³H]NT, [³H]neurotensin; BSA, bovine serum albumin; CDCl₃, deuterated chloroform; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; DLS, dynamic light scattering; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; MBP, *E. coli* maltose-binding protein; MeOD, deuterated methanol; MSP, membrane scaffold protein; MSP1D1, N-terminally truncated membrane scaffold protein 1; NT, neurotensin; NTS1, NT receptor; NTS1_t, NTS1 fusion protein; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol); ROS, rod outer segment; Tev, tobacco etch virus; MS/MS, tandem mass spectrometry.

exchange rates higher by 1 or 2 orders of magnitude in POPC/POPG- and POPG-nanodiscs, respectively, compared to POPC-nanodiscs. Our data demonstrate that negatively charged lipids in the immediate vicinity of a nonvisual GPCR modulate the G-protein-coupling step.

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Introduction

Biological membranes are barriers that separate inner compartments of cells from extracellular environments. Membranes not only provide the physiological environment for membrane proteins but also modulate their function.^{1,2} Membranes contain many different types of lipids³ in relative proportions that depend on the cell type and cellular compartments. Furthermore, changes in membrane lipid composition have been associated with age-related diseases.⁴ Because the properties and geometry of lipid molecules dictate the membrane organization, lipid components directly impact the function of membrane proteins.² This aspect is especially important for membrane proteins that undergo conformational changes as part of their biological activity, as seen for G-protein-coupled receptors (GPCRs).^{5,6} GPCRs are the most abundant superfamily of eukaryotic integral membrane proteins. They initiate signaling events for a wide variety of stimuli by selectively recognizing extracellular ligands and activating intracellular heterotrimeric G proteins at their inner side, causing the release of GDP and the binding of GTP at the $G\alpha$ subunit. $G\alpha$ and $G\beta\gamma$ subunits then dissociate from the receptor and associate with downstream effectors. Arrestins, originally identified as negative regulators of G protein signaling, have been shown to act as adapters that promote distinct intracellular signals in their own right.⁷

Almost all studies on the influence of lipids on GPCR and G protein function have been performed with the visual pigment rhodopsin and its G protein transducin (for review⁸). However, the lipid composition of the rhodopsin containing rod outer segment (ROS) disc membranes is unique and different from that of plasma membranes.^{8,9} The abundance of highly unsaturated acyl chains in its major phospholipids¹⁰ and high fluidity have been suggested to be crucial for anchoring of transducin and for rhodopsin activity. Given the unique lipid environment in which the components for visual signal transduction reside, conclusions inferred from rhodopsin signaling may not simply be transferred to other nonvisual GPCRs.

Neurotensin (NT) is a tridecapeptide (pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) found in both the nervous system and peripheral tissues.^{11,12} NT displays a wide range of biological activities and plays important roles in hypothermia,¹³

antinociception,¹⁴ cancer cell growth,¹⁵ modulation of dopamine neurotransmission,¹⁶ and Parkinson's disease.¹⁷ Three NT receptors, NTS1, NTS2, and NTS3, have been identified; NTS1¹⁸ and NTS2^{19,20} belong to the class A GPCR family, whereas NTS3 (or sortilin) is a member of the Vps10p family, which has a single transmembrane domain.²¹ Most of the known effects of NT are mediated through NTS1,^{22,23} and targeting of NTS1 with synthetic agonists and antagonists for therapeutic purposes has been discussed.²²

NTS1 binds NT at its extracellular surface^{24–26} and couples to a Gq-type G protein at its intracellular surface. These properties distinguish NTS1 from the well-studied rhodopsin and β -adrenergic receptors, which bind small ligands within their transmembrane cores and interact with Gi-type and Gs-type G proteins, respectively. Previously, we have studied the agonist-binding and G protein activation properties of NTS1 monomers and dimers in detergent solution.²⁷ In addition, the propensity of NTS1 to form dimers in a phospholipid bilayer has been investigated.²⁸ However, the effect of lipid molecules on the signaling properties of NTS1 has not yet been addressed.

In the present work, we investigate the effect of lipid charge on NT binding to NTS1 and activation of the Gq protein. Three essential conditions need to be met in order to successfully carry out such studies, namely, (i) the lipid environment surrounding NTS1 has to be controlled with high precision; (ii) NT and the Gq protein must have simultaneous access to the extracellular and intracellular faces of NTS1, respectively; and (iii) the oligomeric state of NTS1 has to be well defined. A number of artificial systems such as vesicles or planar lipid membranes have been used to study GPCRs.²⁹ However, these approaches do not allow for control of receptor oligomerization and homogeneous accessibility of both intracellular and extracellular receptor surfaces. In contrast, the "nanodisc" technology developed by Sligar and colleagues does satisfy all the criteria required for our experiment.³⁰ A nanodisc is composed of two membrane scaffold protein (MSP) molecules stabilizing a planar phospholipid bilayer in which the target membrane protein resides. The nanodisc technique has previously been used to study various aspects of GPCR function.^{31–39} To date, these have not been utilized to investigate the influence of lipid charge on the signaling process.

Here, we report on the incorporation of NTS1 monomers into nanodiscs composed of the zwitterionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), the negatively charged lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), or a mixture of POPC and POPG. We found that NT binding to NTS1 was largely unchanged in these three lipid environments; however, NTS1-catalyzed nucleotide exchange at Gq was markedly increased in the presence of negatively charged lipids.

Results

Nanodisc reconstitution

The purpose of this study was to investigate the effect of phospholipid head group charges on agonist and G protein interaction with the peptide receptor NTS1. All reconstitution experiments were conducted at 4 °C or on ice to preserve NTS1 activity. We utilized the zwitterionic POPC and negatively charged POPG, both of which have phase transition temperatures below 0 °C^{40,41} and a cylindrical molecular shape.⁴² POPG (although a minor phospholipid in eukaryotic membranes⁴²) was chosen instead of the more abundant 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine with a higher transition temperature of 14 °C.⁴³ All nanodisc reconstitution experiments were conducted using zwitterionic POPC, a mixture of POPC/POPG at a molar ratio of 1:1, or negatively charged POPG. First, we established the reconstitu-

tion procedure for nanodiscs without receptor (empty-nanodiscs) in small-scale preparations by optimizing the lipid-to-N-terminally truncated membrane scaffold protein 1 (MSP1D1) ratio and found that a 55:1 molar ratio of lipid to MSP1D1 resulted in well-defined nanodiscs having hydrodynamic radii of 4.7–5.2 nm (Table 1), in agreement with previously published data.³⁰

An overview for NTS1 reconstitution into nanodiscs is shown in Fig. 1. For this, we used the NTS1 fusion protein (NTS1_f)⁴⁴ consisting of the *Escherichia coli* maltose-binding protein (MBP), followed by a tobacco etch virus (Tev) protease recognition site, the rat NTS1, a second Tev protease recognition site at the receptor C-terminus, the *E. coli* thioredoxin (TrxA), and a decahistidine (H10) tag. Based on the above results with empty-nanodiscs, initial reconstitutions with NTS1_f were conducted at a 55:1 lipid:MSP1D1 molar ratio and an excess of MSP1D1 to receptor (110: ~4 MSP1D1:NTS1_f molar ratio) to favor assembly of single NTS1_f molecules.^{34,36} However, this procedure resulted in large particles having diameters of 35–50 nm (data not shown) rather than defined nanodiscs, indicating that the ratio of the lipid to protein components was far from ideal.⁴⁵ Therefore, we optimized the NTS1_f-nanodisc procedure by using lipid:MSP1D1 and MSP1D1:NTS1_f molar ratios of 30:1 and 110:~4, respectively, in the initial reaction setup. After incubation and detergent removal using Bio-Beads, we obtained a mixture of nanodiscs containing NTS1_f (NTS1_f-nanodiscs) and nanodiscs without receptor. The receptor-nanodiscs were purified by subsequent affinity chromatography (Talon resin)

Table 1. Dynamic light scattering and sedimentation velocity experiments

Sample	DLS	Sedimentation velocity			
	$R_{h,1}$ (nm) ^a	$s_{20,w}$ (S) ^b	M_{exp} (kDa) ^b	% load, c (μM) ^c	Lipid ^d ($\epsilon_j/\epsilon_{280}$)
Empty-nanodisc					
POPC	4.7	2.88 ± 0.01	155 ± 8	91 (20)	114
POPC/POPG	5.2	3.22 ± 0.01	174 ± 9	88 (17)	139
POPG	5.1 ± 0.3	3.66 ± 0.02	199 ± 13	91 (20)	102
NTS1 _f -nanodisc					
POPC	6.2 ± 0.5	6.49 ± 0.02	204 ± 6	81 (3.7)	63
POPC/POPG	6.4 ± 0.5	6.66 ± 0.02	211 ± 7	79 (3.6)	105
POPG	6.6 ± 0.8	6.85 ± 0.02	208 ± 5	64 (2.7)	52

^a Data were analyzed in terms of two discrete species (empty-nanodiscs) or a second moment cumulant (NTS1_f-nanodiscs). The hydrodynamic radii ($R_{h,1}$) values were calculated from the average of independent experiments (±SD) (empty-nanodiscs: POPC, $n=1$; POPC/POPG, $n=2$; POPG, $n=2$; NTS1_f-nanodiscs: POPC, $n=3$; POPC/POPG, $n=2$; POPG, $n=2$). In addition to monodisperse species, a minor population of aggregates ($R_{h,2} > 100$ nm) was found in empty-nanodisc (but not in NTS1_f-nanodisc) preparations.

^b Sedimentation coefficients [$s_{20,w}$ (S)] and experimental molecular masses (M_{exp}) represent average values for the major species observed in the continuous $c(s)$ distribution in a single experiment for each nanodisc preparation. Averages and errors (±SD) were obtained from both the absorbance and interference data analyzed using a continuous $c(s)$ distribution in SEDFIT, as well as various hybrid local continuous distributions and global discrete species models in SEDPHAT.

^c Percent of the loading absorbance that represents the major species of interest, based on the best-fit continuous $c(s)$ distribution observed with SEDFIT. The observed concentration c of the major nanodisc species is indicated in parentheses.

^d Lipid stoichiometries per single nanodisc based on the presence of two MSP1D1 molecules and, in the case of the receptor-nanodiscs, one NTS1_f molecule. Stoichiometries are based on signal contributions of the major species to the absorbance (protein alone) and interference (protein and lipid) data.

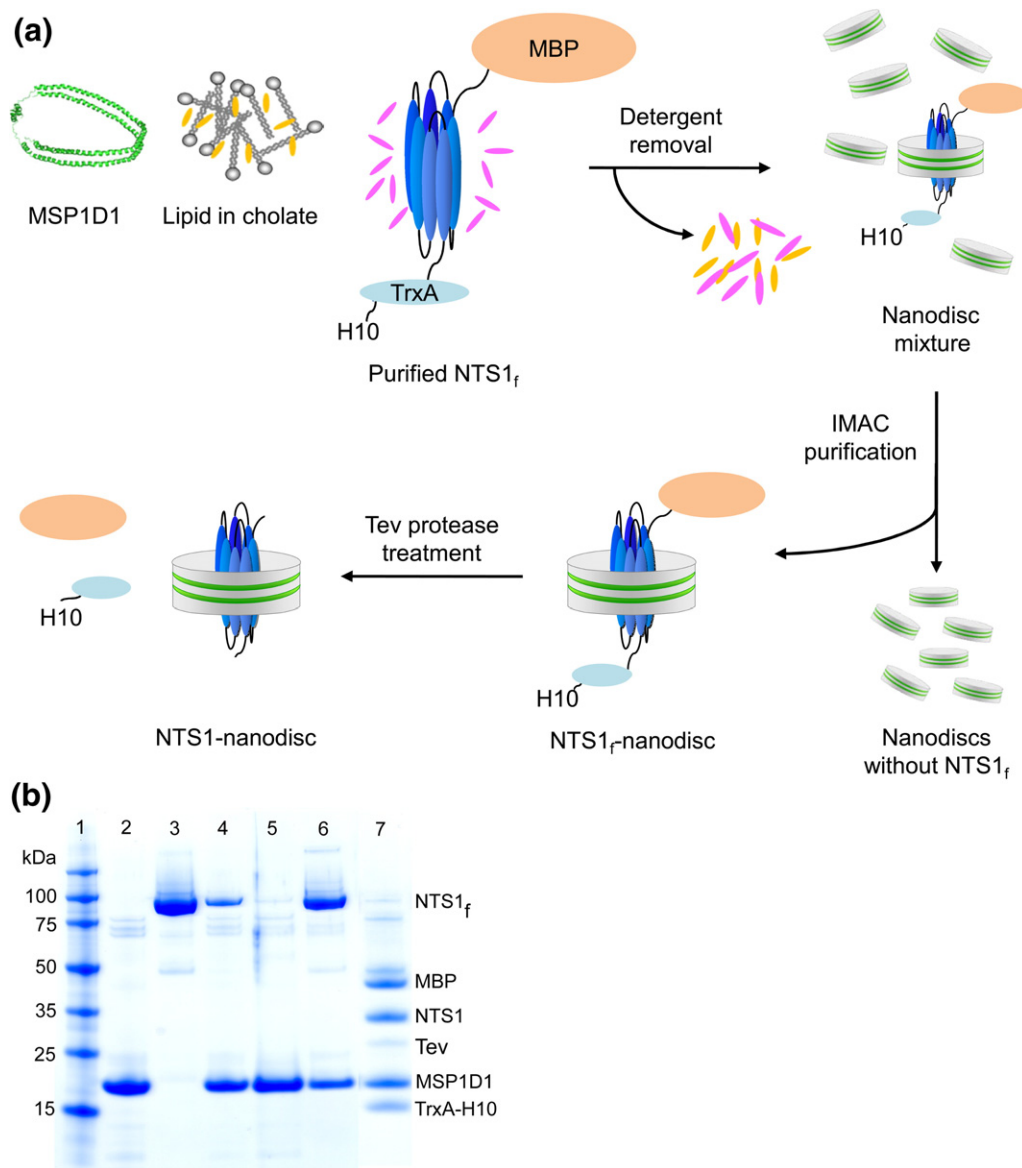


Fig. 1. Preparation of NTS1-nanodiscs. (a) The reconstitution reactions were carried out using MSP1D1, cholate-dissolved lipids, and NTS1_f. After detergent removal, nanodiscs without NTS1_f were separated from NTS1_f-nanodiscs by immobilized metal affinity chromatography exploiting the H10 tail of the receptor fusion protein. Purified NTS1_f-nanodisc was treated with Tev protease prior to ligand binding and nucleotide exchange experiments, to generate the NTS1-nanodisc. (b) Samples of the reconstitution and purification steps were analyzed by SDS-PAGE (4 µg protein/lane). Lane 1, Perfect Protein Markers (15–150 kDa) from Novagen; lane 2, MSP1D1; lane 3, purified NTS1_f; lane 4, mixture after detergent removal by Bio-Beads; lane 5, Talon column flow-through containing nanodiscs without NTS1_f; lane 6, Talon resin eluate containing purified NTS1_f-nanodiscs; lane 7, NTS1-nanodiscs after treatment of NTS1_f-nanodiscs with Tev protease. The SDS-gel shown here was from a reconstitution reaction using POPC. Reconstitutions with POPC/POPG and POPG gave similar results.

exploiting the H10-tag of NTS1_f (Fig. 1). The NTS1_f-nanodiscs, generated with POPC, POPC/POPG, and POPG, had hydrodynamic radii, which were slightly larger than those of the empty-nanodiscs (Fig. 2 and Table 1) and similar to those reported for nanodiscs containing monomeric rhodopsin,^{32,37} β₂-adrenergic receptor,^{35,36} and μ-opioid receptor.³⁴

A single NTS1 is reconstituted into receptor-nanodiscs

To determine whether a single monomeric NTS1_f was reconstituted in the receptor-nanodiscs, we characterized NTS1_f and empty-nanodisc preparations by dynamic light scattering (DLS), sedimen-

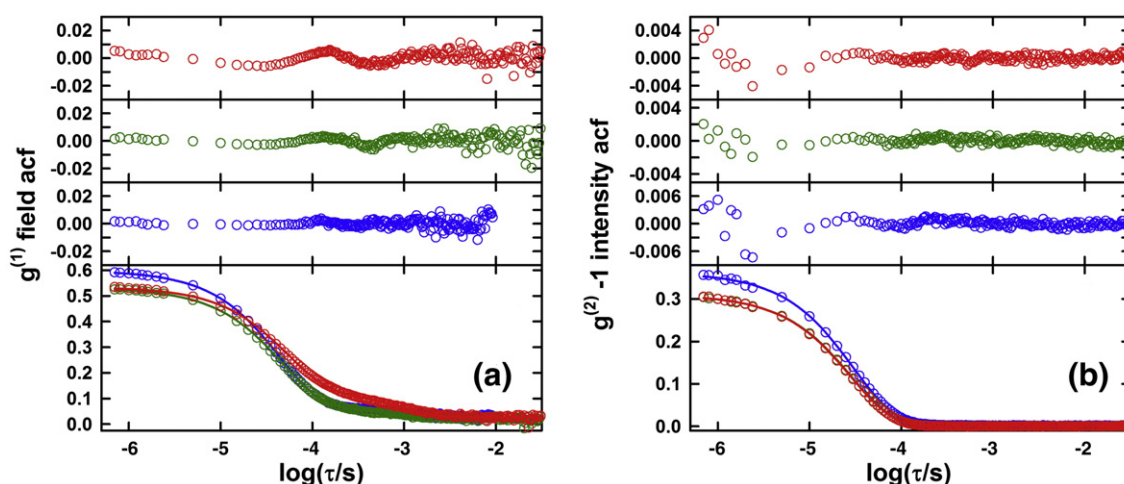


Fig. 2. DLS analysis of nanodiscs. (a) DLS autocorrelation functions for empty-nanodiscs reconstituted with POPC (blue), POPC/POPG (green), and POPG (red) are best modeled in terms of contributions from two discrete species corresponding to empty-nanodiscs ($R_{h,1}$) and traces of aggregates ($R_{h,2}$). The autocorrelation function is shown along with the best-fit two-species model and corresponding residuals. (b) DLS autocorrelation functions for NTS1_F-nanodiscs prepared using POPC (blue), POPC/POPG (green), and POPG (red) are best modeled in terms of a paucidisperse species. The autocorrelation function for a batch of NTS1_F-containing POPC-MSP1D1 nanodisc is shown along with the best fit using a quadratic cumulant and corresponding residuals. Data for these preparations of the POPC/POPG- and POPG-nanodiscs practically superimpose.

tation velocity, and lipid analyses. First, we characterized empty-nanodiscs, reconstituted using MSP1D1 and various lipids, by a combination of sedimentation velocity and DLS in order to establish the validity of these methods. Sedimentation velocity experiments indicated the presence of a major species at 2.9–3.7 S, depending on the lipid

composition (Fig. 3a and Table 1). These observations are in agreement with DLS studies that indicate the presence of a major species having a hydrodynamic radius ($R_{h,1}$) of ~5 nm (Fig. 2a and Table 1), along with minute quantities of a larger aggregate ($R_{h,2} > 100$ nm). We note that the sedimentation coefficient of the empty-nanodisc recon-

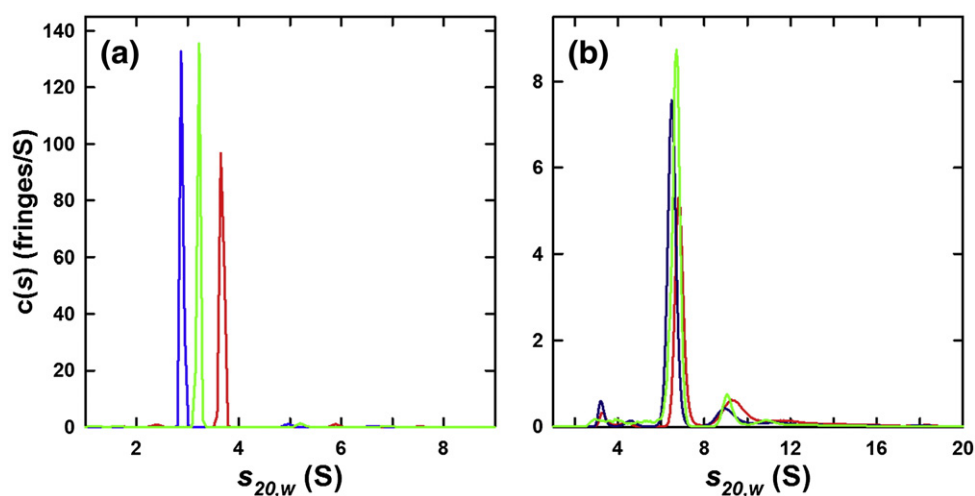


Fig. 3. Sedimentation velocities analysis of nanodiscs. (a) Sedimentation velocity $c(s)$ distributions obtained in SEDFIT for empty-nanodiscs reconstituted with POPC (blue), POPC/POPG (green), and POPG (red) based on interference data. (b) $c(s)$ distributions obtained in SEDFIT for NTS1_F-nanodiscs reconstituted with POPC (blue), POPC/POPG (green), and POPG (red) based on interference data. Besides the major species, a small contribution is observed at lower and higher S values, possibly representing small amounts of free MSP1D1 or empty-nanodiscs, and aggregated NTS1_F-nanodiscs, respectively. Data were collected at 40 krpm and 10.0 °C.

stituted using combinations of POPC and POPG increases with increasing POPG content, reflecting in part the increased molecular mass of this lipid and possibly a smaller partial specific volume for POPG. The best-fit frictional ratios f/f_0 of 1.2–1.3 obtained from the $c(s)$ analysis are consistent with the overall shape of the empty-nanodiscs; however, we find that the best-fit molecular masses for the major species are larger than expected based on a lipid: MSP1D1 ratio of 55:1. In a manner analogous to methods developed for the study of membrane proteins in the presence of solubilizing detergents,^{46,47} we combined the absorbance and interference signal intensities for the major species to determine the lipid-to-protein content (Table 1). Briefly, the absorbance signal for the species of interest, obtained from an integration of the $c(s)$ distribution, was used to determine the MSP1D1 concentration. The corresponding contribution of MSP1D1 to the interference signal is then used to determine the lipid concentrations. We found that stoichiometries of 102–139 lipid molecules per empty-nanodisc were consistent with expectations⁴⁵ and note that these determinations are based solely on the signal intensities and made independent of any assumptions regarding the molecular mass and partial specific volumes.

As in the case of the empty-nanodiscs, the purified NTS1_f-nanodiscs were characterized using a combination of analytical ultracentrifugation and DLS. Sedimentation velocity experiments showed the presence of a major species of 6.5–6.9 S depending on the lipid composition (Fig. 3b and Table 1) and a small amount of higher mass impurities (~9 S) (Fig. 3b). These observations are in broad agreement with DLS studies that indicate the presence of species having an average hydrodynamic radius of 6.2–6.6 nm (Fig. 2b and Table 1). DLS studies further indicate that the higher mass impurities present appear to be preparation dependent—for example, three separate preparations of POPC receptor-nanodiscs lead to best-fit average hydrodynamic radii of 5.7, 6.3, and 6.7 nm. The corresponding polydispersity indices (μ_2/Γ_2) increased with the hydrodynamic radius, reflecting an increasing contribution from the higher-mass species (data not shown). Even though DLS does not resolve the contributions from the various species, sedimentation velocity does, allowing us to characterize the major receptor-nanodisc complex. Molecular masses for the major species, derived from the best-fit frictional ratio f/f_0 of 1.3–1.4, are consistent with a monomer NTS1_f-nanodisc (Table 1). To further confirm that a single NTS1_f molecule is reconstituted into the 6.5–6.9 S nanodiscs, we used the absorbance and interference signal contributions of the major species to determine the lipid stoichiometry. We found 52–105 lipid molecules per NTS1_f-nanodisc depending on the lipid composition (Table 1), in

broad agreement with determinations of the lipid content by HPLC/tandem mass spectrometry (MS/MS) and ¹H NMR (Table 2). The lipid numbers determined by ¹H NMR (Table 2) may represent a slight underestimate due to the techniques involved in sample preparation (see Materials and Methods). In addition, we note that the observed higher lipid content of NTS1_f-POPC/POPG-nanodiscs (Table 1) is possibly an outlier (one sedimentation velocity experiment was performed for each nanodisc preparation) as the lipid content determined by ¹H NMR is similar for all three types of receptor-nanodiscs (Table 2).

These observations support the presence of one NTS1_f molecule and two MSP1D1 molecules per receptor-nanodisc; they do not support the presence of two NTS1_f molecules in a single nanodisc. In the determination of the lipid content using the absorbance and interference signal intensities (Table 1), we assumed the presence of two MSP1D1 and one NTS1_f per nanodisc. Assuming the presence of two NTS1_f molecules per nanodisc leads to calculated lipid stoichiometries (POPC, 133 lipid molecules per nanodisc; POPC/POPG, 210; POPG, 120) that exceed that of the empty-nanodiscs (Table 1). As the hydrodynamic radii of the NTS1_f-nanodiscs reflect the z-average of the preparation (major species and a small amount of higher mass impurities), the radii of the major species are expected to be slightly smaller than the values reported. As the excess amount of lipid and NTS1_f would be difficult to accommodate into a nanodisc just slightly larger than an empty-nanodisc, it is unlikely that these nanodiscs contain more than a single NTS1_f.

Furthermore, simple volume and shape calculations^{48,49} based on an f/f_0 of 1.35 return an expected sedimentation coefficient of 6.45 S for a receptor-nanodisc having a stoichiometry of 60:2:1

Table 2. Determination of nanodisc lipid content by HPLC/MS/MS and ¹H NMR

	HPLC/MS/MS	¹ H NMR
Empty-nanodisc		
POPC	141	95 ^a
POPC/POPG	n.d.	85 ^a
POPG	n.d.	92 ^a
NTS1 _f -nanodisc		
POPC	66	55 ^a
POPC/POPG	n.d.	44 ^a
POPG	n.d.	67 ^a

n.d., not determined.

The number of lipid molecules per empty-nanodisc is based on the presence of two MSP1D1 molecules and, in the case of the receptor-nanodiscs, one NTS1_f molecule.

^a The lipid content by ¹H NMR may represent a slight underestimate due to potential sample losses during the filtration step required for sample preparation. The data on lipid content from sedimentation velocity experiments are shown in Table 1.

POPC:MSP1D1:NTS1_f (see Table 1). Corresponding calculations made for a dimeric receptor-nanodisc with a stoichiometry of 10:2:2 (whereby we assumed that 1 NTS1_f molecule displaces 50 lipid molecules³²) returns a sedimentation coefficient of 10.1 S. Based on this and the experimental lipid stoichiometry as estimated from the absorbance and interference signal intensities, the higher mass impurities observed at ~9 S (Fig. 3b) are unlikely to represent receptor-nanodiscs containing two NTS1_f molecules. Importantly, the calculated *S* value of 10.1 contrasts with the experimentally determined *S* values of 6.5–6.9 for the major species, suggesting that the NTS1_f nanodiscs contain only one receptor molecule.

Negatively charged lipids modulate nucleotide exchange at Gαq, but not NT binding to NTS1

NTS1_f-nanodiscs were treated with Tev protease prior to ligand binding and nucleotide exchange experiments, to remove the MBP and TrxA-H10 tags and thus generate NTS1 with near-authentic N- and C-termini (Figs. 1 and 4).

To assess whether agonist binding to NTS1 is affected by the various lipid compositions used for the nanodisc preparations, we conducted [³H] neurotensin ([³H]NT) saturation binding and [³H] NT/NT homologous competition experiments in the absence of G protein (representative experiments are shown in Fig. 5). Data from saturation experiments were best fit to a one-site binding equation; the corresponding *K_d* values for POPC, POPC/POPG, and POPG were similar (Table 3). Likewise, the data from homologous competition experiments were best fit to a sigmoidal dose–response equation with a standard slope of 1, and the corresponding IC₅₀ values were also comparable. The calculated *K_i*

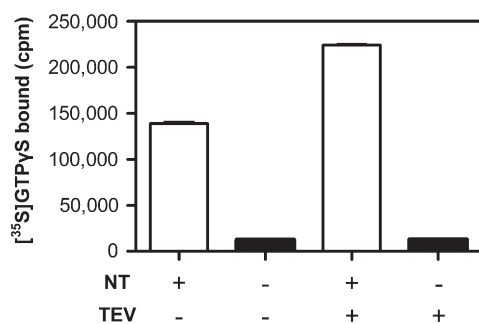


Fig. 4. Nucleotide exchange at Gαq requires a near-authentic receptor C-terminus. GDP/[³⁵S]GTPγS exchange experiments were conducted with POPC-nanodiscs containing NTS1_f in the presence and absence of Tev protease. Increased [³⁵S]GTPγS binding was observed after cleaving off the C-terminal affinity tag. Two similar experiments in duplicate were performed for each test; one representative experiment is shown.

values matched the corresponding *K_d* values obtained from saturation binding experiments. To assess whether agonist binding to NTS1 in the presence of Gq protein is affected by the lipid composition, we conducted dose–response experiments (NT saturation of NTS1-catalyzed GDP/GTPγS exchange, Fig. 5d–f) by varying the concentration of NT and recording nucleotide exchange at Gαq. Consistent with the homologous competition experiments, the EC₅₀ values for NT-stimulated activation of Gαq by NTS1 were also similar and independent of the lipid composition (Table 3). Altogether, these results suggest that the nanodisc lipid compositions did not substantially modulate agonist binding to NTS1.

In contrast to NT binding, G protein activation was dramatically affected by the nature of the lipid present in the respective nanodiscs (Fig. 6 and Table 3). Even though Gαq (but not Gβ₁γ₁) is prepared using the detergent 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (Chaps), the integrity of the nanodiscs is not affected by this as the highest Gαq concentration used in the nucleotide exchange assays was 1 μM, thus limiting the free Chaps concentration in the exchange reactions to less than 1/10th of its critical micellar concentration.⁵⁰

The Gαq saturation experiments of NTS1-catalyzed nucleotide exchange were carried out by holding the Gβ₁γ₁, NT, and NTS1 concentrations constant (at 1 μM, 10 μM, and 5 nM, respectively) and varying the concentration of Gαq (Fig. 6a). For NTS1-POPG-nanodiscs, we determined a robust *K_m* (Gαq) value in the sub-micromolar range (Table 3). A *K_m* value could not be reliably calculated for the NTS1-POPC/POPG-nanodiscs because of the low affinity. Similarly, we were unable to determine a *K_m* value for the NTS1-POPC-nanodiscs because the exchange reactions were too slow to measure under our experimental conditions. Gβ₁γ₁ saturation experiments of NTS1-catalyzed nucleotide exchange were done by holding the concentrations for Gαq, NT, and NTS1 at 250 nM, 10 μM, and 5 nM, respectively, and varying the concentration of Gβ₁γ₁ (Fig. 6b). The *K_m* (Gβ₁γ₁) value for POPG was found to be 3-fold lower than that for POPC/POPG (Table 3). As for Gαq, the *K_m* (Gβ₁γ₁) for NTS1-POPC-nanodiscs could not be determined because the reactions were too slow. Taken together, our data correlate an apparent affinity increase with the presence of POPG in NTS1-nanodiscs. The effect of the negatively charged lipid POPG on the NTS1-catalyzed nucleotide exchange was seen even clearer when normalizing nucleotide exchange rates at the highest Gαq (1 μM), Gβ₁γ₁ (5 μM), and NT (1 μM) concentrations used in the respective Gαq (Fig. 6a), Gβ₁γ₁ (Fig. 6b), and NT (Fig. 5d–f) saturation assays {catalyzed [³⁵S]GTPγS bound in cpm (nM NTS1 protein)⁻¹ min⁻¹}. Compared to NTS1-POPC-

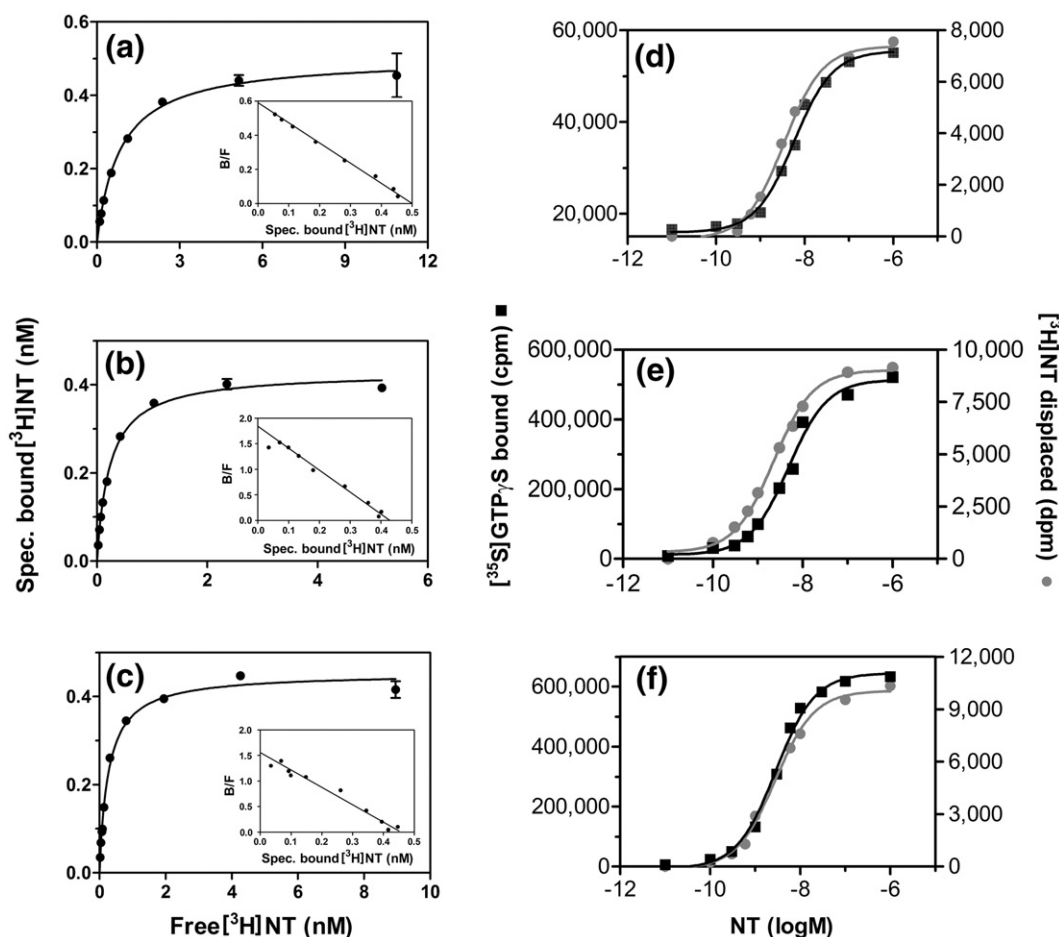


Fig. 5. Pharmacological properties of NTS1 in nanodiscs. (a–c) Saturation binding experiments: $[^3\text{H}]\text{NT}$ binding to NTS1 incorporated into nanodiscs prepared using POPC (a), POPC/POPG (b), and POPG (c). A one-site binding equation was used for curve fitting. (Inset) Scatchard transformation with $B/F = \text{bound}/\text{free}$. (d–f) Homologous competition and agonist-stimulated activation of $\text{G}\alpha\text{q}$: $[^3\text{H}]\text{NT}/\text{NT}$ competition experiments in the absence of G protein are shown for NTS1-nanodiscs prepared with POPC (d), POPC/POPG (e), and POPG (f) (● (gray circles), total NT added (x -axis) versus total $[^3\text{H}]\text{NT}$ displaced (right y -axis)). Agonist-stimulated activation of $\text{G}\alpha\text{q}$: $[^3\text{S}]\text{GTP}\gamma\text{S}$ binding was recorded in response to the indicated amounts of NT in the presence of $\text{G}\alpha\text{q}$ and $\text{G}\beta_1\gamma_1$ (■, total NT added (x -axis) versus total $[^3\text{S}]\text{GTP}\gamma\text{S}$ bound (left y -axis)). Data were best fit to equations with a Hill slope of 1. Note that the nucleotide exchange reactions proceeded for 120 min, 45 min, and 15 min in the case of POPC-, POPC/POPG-, and POPG-nanodiscs, respectively. One representative experiment for each is shown.

nanodiscs, normalized nucleotide exchange rates are increased in all cases by 1 and 2 orders of magnitude for NTS1-POPC/POPG- and NTS1-POPG-nanodiscs, respectively (Table 3). Likewise, normalization of the data in Fig. 6a and b emphasized the effect of POPG (Fig. 6c and d). The results clearly indicate that the Gq protein apparent affinity to NTS1 and nucleotide exchange rates increase in the presence of negatively charged lipids.

Discussion

The importance of phospholipids for the conformational energetics of rhodopsin and activation of transducin has been well documented.^{8,51–57} How-

ever, rhodopsin represents an unusual case with respect to receptor–lipid interactions,⁸ and lessons learned from rhodopsin signaling may not be simply extrapolated to other GPCRs. Here, we investigated the effect of lipid head group charges on the signal transduction properties of the Gq-coupled peptide receptor NTS1 embedded into nanodiscs containing the zwitterionic lipid POPC or the negatively charged lipid POPG or a mixture of the two. We show that an increasing negative charge density dramatically affects the NTS1-catalyzed GDP/GTP γS nucleotide exchange rates for $\text{G}\alpha\text{q}$, whereas agonist binding shows little influence by the surrounding lipid type. Of interest, it has been shown that the interaction of arrestin with rhodopsin, reconstituted into nanodiscs, was enhanced in

Table 3. Pharmacological properties of NTS1 in nanodiscs

	POPC	POPC/POPG	POPG
K_d (nM)	1.04 ± 0.46 ($n=3$)	0.24^a ($n=2$)	0.31 ± 0.11 ($n=3$)
K_i (nM)	1.16 ± 0.02 ($n=4$)	0.24 ± 0.04 ($n=4$)	0.35 ± 0.03 ($n=3$)
IC_{50} (nM)	3.46 ± 0.07 ($n=4$)	2.34 ± 0.36 ($n=4$)	2.66 ± 0.24 ($n=3$)
EC_{50} (nM)	4.92 ± 1.33 ($n=3$)	4.54 ± 0.94 ($n=3$)	3.05 ± 0.41 ($n=3$)
K_m Gαq (μM)	n.d. ($n=2$)	>5 ($n=3$)	0.55 ± 0.07 ($n=3$)
K_m Gβ ₁ γ ₁ (μM)	n.d. ($n=2$)	8.2 ± 4.3 ($n=4$)	2.40 ± 0.12 ($n=4$)
Normalized data [catalyzed [³⁵ S]GTPγS bound (cpm) (nM NTS1 protein) ⁻¹ min ⁻¹]			
Gαq saturation	21^b ($n=2$)	656 ± 166 ($n=3$)	3496 ± 50 ($n=3$)
Gβ ₁ γ ₁ saturation	11^c ($n=2$)	280 ± 174 ($n=4$)	2784 ± 170 ($n=4$)
NT saturation	45 ± 30 ($n=3$)	2177 ± 548 ($n=3$)	7392 ± 1288 ($n=3$)

All values are given $\pm$ SD. K_d , equilibrium dissociation constant (saturation binding); IC_{50} , half maximal inhibitory concentration (homologous competition); K_i , dissociation constant calculated by the Cheng and Prusoff equation; EC_{50} , half maximal effective concentration (NT saturation of Gαq activation); K_m , binding constants for Gαq and Gβ₁γ₁ (Gαq and Gβ₁γ₁ saturation); normalized data from Gαq, Gβ₁γ₁, and NT saturation experiments on ice given as catalyzed [³⁵S]GTPγS bound (cpm) (nM NTS1 protein)⁻¹ min⁻¹ are the average from the respective experiments. n , number of independent experiments; n.d., not determined.

^a Average value of 0.21 and 0.26 nM.

^b Average value of 17 and 25 catalyzed [³⁵S]GTPγS bound (cpm) (nM NTS1 protein)⁻¹ min⁻¹.

^c Average value of 8 and 13 catalyzed [³⁵S]GTPγS bound (cpm) (nM NTS1 protein)⁻¹ min⁻¹.

the presence of phospholipids with acidic head groups.^{58,59}

The following observations support the notion that NTS1-nanodiscs contain a single receptor molecule. NTS1_f was prepared in buffer containing detergent at concentrations that render the receptor monomeric before reconstitution.^{27,44} The initial reconstitution mixture contained an excess of MSP1D1 over NTS1_f,

which favors the incorporation of a single receptor molecule per nanodisc.^{34–37} Sedimentation velocity data and lipid analyses support the presence of one NTS1_f molecule and two MSP1D1 molecules per receptor-nanodisc, but not two receptor molecules. Furthermore, data from [³H]NT saturation and competition experiments were best fit to equations with a Hill slope of 1, as opposed to Hill slopes of 2

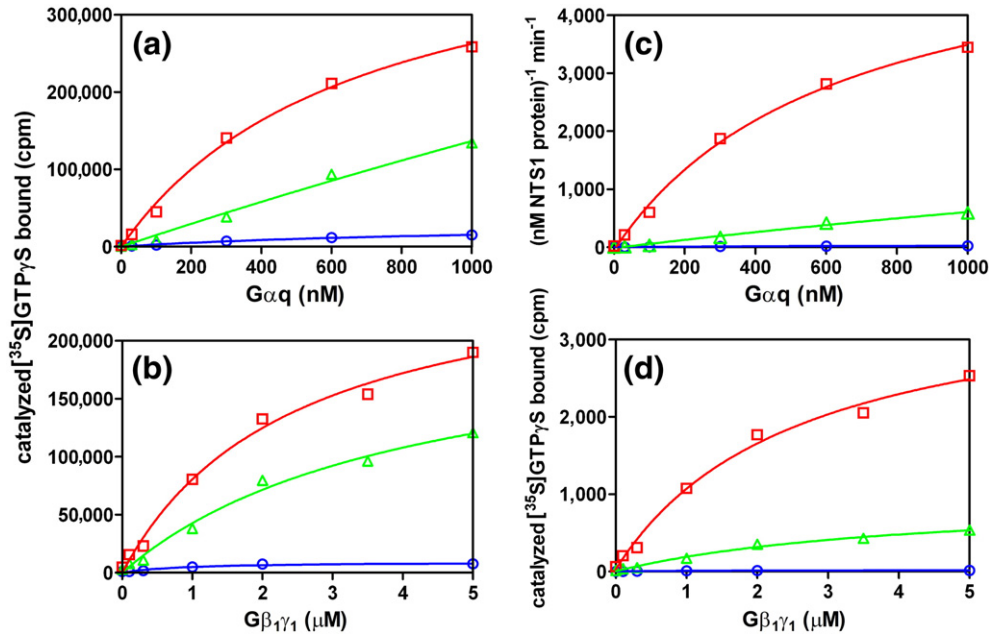


Fig. 6. Gαq (a) and Gβ₁γ₁ (b) saturation of NTS1-catalyzed GDP/GTPγS exchange. [³⁵S]GTPγS binding was measured in reactions containing NTS1-nanodiscs with POPC (blue), POPC/POPG (green), and POPG (red), respectively. The fractional contribution of non-catalyzed nucleotide exchange at a given Gαq or Gβ₁γ₁ concentration was estimated in the absence of NT and subtracted from total [³⁵S]GTPγS binding. Normalization of the data to catalyzed [³⁵S]GTPγS bound (cpm) (nM NTS1 protein)⁻¹ min⁻¹ [Gαq (c) and Gβ₁γ₁ (d) saturation] emphasizes the effect of POPG on the nucleotide exchange reaction. One representative experiment for each is shown.

we have previously observed for dimeric NTS1 in detergent solution,²⁷ consistent with one NTS1 molecule per nanodisc.

The experimentally determined average lipid content in NTS1_f-nanodiscs is ~65 molecules (Tables 1 and 2), likely providing one layer of lipid molecules around NTS1. A similar number was found for rhodopsin in ROS membranes (65–75 phospholipids per rhodopsin monomer^{60,61}). The lipid content in NTS1-nanodiscs is lower than that found in nanodiscs containing monomeric rhodopsin (196 POPC molecules, determined by phosphate analysis³²). This difference is likely due to the use of the smaller MSP1D1 in our study, rather than the MSP1E3D1 utilized in the rhodopsin study,³² which leads to the formation of a larger disc and hence incorporation of more lipid.⁶² As in our studies, Leitz *et al.* report the presence of ~100 POPC molecules for a β_2 -adrenergic receptor-nanodisc constructed with MSP1.³⁵ Note that other reports on GPCR-nanodiscs^{31,33,34,36,37} state the lipid:MSP: receptor ratios in the initial reaction mixtures but do not report an experimentally determined lipid content for the purified receptor-nanodiscs.

Both POPC (a zwitter-ionic lipid with neutral electrical surface potential) and POPG (negatively charged) are classified as cylindrical in shape;⁴² hence, the observed effects must relate to differences in charge properties rather than to their packing within the NTS1-nanodiscs. The agonist binding and nucleotide exchange experiments were conducted at 0–4 °C. Although pure POPC and POPG have transition temperatures below 0 °C (POPC, –2.6 °C;⁴¹ POPG, –4.0 °C⁴⁰), we have no experimental data on the phase behavior of POPC and POPG in NTS1-nanodiscs. A study of the phase transition properties of the lipids di-palmitoyl-phosphatidylcholine and di-myristoyl-phosphatidylcholine in empty-nanodiscs revealed a shift of the transition midpoint to higher temperature compared to vesicles, reflecting a more ordered state of the lipids in close contact with MSP compared to “core” lipids.^{63,64} Hence, the close contact of POPC and POPG with MSP1D1 and NTS1 may well alter their properties.

Studies of lipid influence on rhodopsin signaling have suggested a number of mechanisms including bilayer curvature elastic stress, dynamics of annular lipids, membrane thickness, rhodopsin–rhodopsin interactions, electric membrane surface potential, and cholesterol concentration.⁵⁶ In our study, we can eliminate NTS1 dimerization and bilayer elastic stress as an explanation as our nanodiscs contain one NTS1 molecule, surrounded by one layer of lipid. In addition, the rapid exchange of annular lipids with bulk lipid observed in vesicles⁵⁶ is not given in nanodiscs. Hence, the strong effect of the negatively charged POPG on G protein affinity and nucleotide exchange rates may arise from a combi-

nation of the following mechanisms: partitioning of G protein components to the receptor-nanodiscs, specific interactions between lipid and receptor residues, and local net charges at the NTS1/lipid interface.

Upon combining NTS1-nanodiscs with G protein components, the assembly mixture contains G protein heterotrimers, excess $G\beta_1\gamma_1$, NTS1, and no free $G\alpha_q$. Initial binding of $G\alpha_q/G\beta_1\gamma_1$ and $G\beta_1\gamma_1$ to receptor-nanodiscs occurs under conditions of rapid equilibrium. After addition of the GDP/[³⁵S]GTP γ S mix, $G\alpha_q$ with bound [³⁵S]GTP γ S dissociates from NTS1 and $G\beta_1\gamma_1$. At this point in the exchange reactions, the precise stoichiometries of $G\alpha_q$ and $G\beta_1\gamma_1$ bound to the receptor are unknown, as our exchange assays measure turnover rates, not stoichiometries. Therefore, we cannot at this time strictly allocate the observed effects of lipid charge to partitioning of $G\alpha_q/G\beta_1\gamma_1$ and $G\beta_1\gamma_1$ to nanodiscs or direct activation of $G\alpha_q/G\beta_1\gamma_1$ by NTS1, despite discussing these aspects separately.

It has been found that membrane association of the transducin heterotrimer with negatively charged phosphatidylserine vesicles occurs with somewhat lower affinity compared to partitioning into phosphatidylcholine vesicles,^{65,66} suggesting that negative charge is not a major driving force for the partitioning of the $G\alpha_q/G\beta_1\gamma_1$ heterotrimer to NTS1-nanodiscs. In contrast, membrane association of $G\beta_1\gamma_1$ is driven in part by electrostatic effects^{67–69} (and by hydrophobic effects via the farnesyl modification at the C-terminus of the $G\gamma_1$ subunit), and a modest increase in $G\beta_1\gamma_1$ binding to model membranes containing negatively charged lipids has been reported.^{65,66} Overall, the effect of lipid charge on the partitioning of G protein components to the receptor-nanodiscs seems not sufficient to solely explain the observed increase in nucleotide exchange rates.

Recently, specific interactions between lipids and protein residues have been suggested to influence membrane protein function¹ with lipid molecules binding into clefts between helices, affecting the way helices pack into bundles. Hypothetically, such lipid-induced conformational changes in GPCRs (as opposed to the effect of bilayer curvature elastic stress) could affect G protein activation. It has been proposed that the molecular properties of lipids in the annular lipid layer surrounding rhodopsin are important for the MI–MII equilibrium,⁷⁰ and evidence for the specific interaction of lipid with rhodopsin in different conformational states has been reported.^{71–74} Furthermore, a cholesterol-binding site has been established for the β_2 -adrenergic receptor.⁷⁵ In the case of NTS1, additional experiments are needed to determine whether POPG triggers conformational changes.

The actual surface potential at the NTS1/lipid interface is unknown because bivalent cations,

present in the nucleotide exchange reaction, interact with the negatively charged lipid phosphate groups and because the concentration of Na^+ possibly changes with increasing amounts of POPG. In addition, it has been proposed that a negative surface potential leads to accumulation of hydronium ions.⁵⁷ This may favor the protonation of Glu166 of the conserved E(D)RY motif in NTS1 and hence the productive interaction with the G protein^{56,76–78} in POPG-containing NTS1-nanodiscs.

In conclusion, our studies carried out under carefully controlled experimental conditions with the Gq-coupled peptide receptor NTS1 show that the overall conformation of the NT binding site is little influenced by the lipid type, while the lipid charge in the immediate vicinity of NTS1 exerts a strong influence on the Gq protein interaction. As the lipid composition differs from tissue to tissue and even within a single cell membrane, variation of lipids in the immediate vicinity of NTS1 may provide a mechanism to regulate cell-specific NTS1 signaling in addition to desensitization and downregulation.

Materials and Methods

Materials

The tritiated agonist [^3H]NT {[3,11-tyrosyl-3,5- ^3H (N)]-pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu} was purchased from PerkinElmer. Unlabeled NT was synthesized by the Center for Biologics Evaluation and Research (Food and Drug Administration, Bethesda, MD, USA). The detergents *n*-dodecyl- β -D-maltopyranoside, Chaps, sodium cholate, and cholesteryl hemisuccinate Tris salt were obtained from Anatrace. The lipids POPC and POPG were purchased from Avanti Polar Lipids. The Tev protease mutant His-Tev(S219V)-Arg was essentially prepared as reported.^{44,79}

NTS1 expression and purification

The rat NTS1 fusion protein (NTS1_f) (MBP-N10-Tev-rT43NTR-CH2-N5G3S-G3S-TrxA-H10) consists of MBP, followed by a linker and a Tev protease recognition site, the N-terminally truncated rat NTS1 starting at Thr43, a second Tev protease recognition site at the receptor C-terminus, a linker, TrxA, and an H10-tag. NTS1_f was produced in *E. coli* and purified as described previously.⁴⁴ To obtain receptor devoid of MBP and affinity tag, we incubated NTS1_f with Tev protease to generate NTS1 with Ser-Gly-Ser at the N-terminus and with the C-terminus ending in Glu421-Asn-Leu-Tyr-Phe-Gln.

Preparation of Gαq and Gβ₁γ₁

Cephalopod Gαq was purified from dark-adapted retinas of *Sepia officinalis* as described previously.⁸⁰ The final Gαq concentration was 10 μM in a buffer containing 10 mM Mops (pH 7.4), 100 mM NaCl and 4 mM Chaps.

The heterodimer of bovine transducin G_tβγ (Gβ₁γ₁) was purified as follows. Bovine ROS discs were isolated from frozen dark-adapted bovine retina obtained from W. Lawson, Inc. by a modification of the sucrose density gradient method of Papermaster and Dreyer.⁸¹ All solutions used were as described previously,⁸² but instead of layering the 45% sucrose-floated outer segment fraction on multistep gradients, the ROS disc fraction was isolated by two sequential single-step gradients. Briefly, the pelleted outer segment fraction was resuspended in a 34% sucrose solution and adjusted to 34% sucrose with a 60% sucrose solution. Twenty-five milliliters of that suspension was overlaid with 10 ml of a 25% sucrose solution for centrifugation at 27,000 rpm for 30 min in a Beckman SW28 rotor. The 26%/34% interface was harvested, diluted with 1.5 volumes of PEMD buffer [10 mM sodium phosphate (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM MgCl₂, and 1 mM dithiothreitol (DTT)] and collected by sedimentation for 30 min at 20,000 rpm in a Beckman JLA25 rotor. The pellets were resuspended in a 26% sucrose solution and adjusted to 26% sucrose with 60% sucrose, and 20 ml was layered over 15 ml of 30% sucrose for centrifugation at 27,000 rpm for 30 min in an SW28 rotor. The 26%/30% interface was collected by dilution with 1.5 volumes of PEMD buffer and sedimented for 30 min at 20,000 rpm in a JLA25 rotor. The final ROS disc pellet was resuspended with 12% sucrose in PEMD buffer for storage at −80 °C.

Transducin was isolated from light-exposed ROS disc membranes by the method of Kuhn *et al.*,⁸³ and G_tα and Gβ₁γ₁ were resolved by chromatography over Affigel-Blue Agarose by a modification of the method of Yamazaki *et al.*⁸⁴ Briefly, instead of collecting sequential washes of increasing NaCl concentrations, the GTP-eluted transducin heterotrimer was applied to a 25-ml bed-volume column of Affigel-Blue (Bio-Rad) and was eluted with a segmented gradient of NaCl (100–300 mM NaCl for 8 bed volumes, 300–1000 mM NaCl for 3 bed volumes). The bulk of the G_tα was adsorbed and eluted at 500 mM NaCl, free of Gβ₁γ₁. The heterodimer Gβ₁γ₁ was largely found in the flow-through, and residual contaminating G_tα was removed by re-chromatography with the same procedure. The final Gβ₁γ₁ concentration was 25 μM in buffer containing 10 mM MOPS and 1 mM DTT.

Preparation of MSP1D1

The expression plasmid pMSP1D1 (# 20061, Addgene) is a derivative of pET28a and codes for the synthetic gene of the deletion mutant (Δ1–54) of the human Apolipoprotein A-I, preceded by an N-terminal heptahistidine (H7) tag, spacer sequence, and Tev protease recognition site (H7-MSP1D1).⁶² Expression was carried out as described⁶² with modifications. BL21Gold(DE3) cells (Stratagene) harboring pMSP1D1 were grown at 37 °C in double-strength TY medium containing 50 μg/ml kanamycin to an OD₆₀₀ of 4. After induction with 0.3 mM isopropyl- β -D-thiogalactopyranoside, the temperature was decreased to 28 °C. The cells were harvested 4 h later, frozen in liquid nitrogen, and stored at −80 °C until further use.

For purification, cells were resuspended with buffer A (50 mM Tris-HCl, pH 7.4, and 200 mM NaCl) and then lysed twice with a French Press. Cell debris was removed by centrifugation (Beckman 70Ti rotor, 55,000 rpm,

45 min, 4 °C). Imidazole was added to the supernatant to a final concentration of 25 mM. The sample was passed through a 0.2- μ m filter (Stericup; Millipore) and loaded onto a Ni-NTA column (Qiagen), equilibrated with buffer B (buffer A containing 25 mM imidazole). The resin was washed with buffer B, and H7-MSP1D1 was eluted with buffer C (buffer A containing 280 mM imidazole). Fractions containing H7-MSP1D1 were identified by SDS-PAGE (NuPAGE 4–12% 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol gel, 1 $\times$ 2-(4-morpholino)ethanesulfonic acid running buffer; Invitrogen). Pooled fractions containing H7-MSP1D1 were concentrated using a Centriprep YM-10 device (Millipore); imidazole was then removed by a desalting step (PD10 column, equilibrated with buffer A; GE Healthcare). Purified H7-MSP1D1 was frozen in liquid nitrogen and stored at –80 °C until further use. The protein concentration was determined by measuring the absorbance at 280 nm with a NanoDrop 1000 spectrophotometer (version 3.6.0; Thermo Scientific) using a calculated extinction coefficient of 21,430 M^{–1} cm^{–1} and a calculated molecular mass of 24,793 Da (ProtParam, ExPASy†). The protein content was also determined by the method of Schaffner and Weissmann⁸⁵ with bovine serum albumin (BSA) as the standard. The identity of H7-MSP1D1 was confirmed by N-terminal sequencing (GHHHHHHHDYDIPTTENLYFQGSTF).

Prior to reconstitution, the H7-tag was removed from H7-MSP1D1 by incubation with Tev protease (150:1 M/M H7-MSP1D1/Tev protease) at room temperature for 1.5 h. The sample was then incubated with Talon resin (Clontech Laboratories) for 1 h at 4 °C. MSP1D1 was recovered from the flow-through and the subsequent wash with buffer D (buffer A containing 20 mM imidazole). The protein concentration was determined by measuring the absorbance at 280 nm with a NanoDrop 1000 spectrophotometer using a calculated extinction coefficient of 18,450 M^{–1} cm^{–1} and a calculated molecular mass of 22,044 Da. MSP1D1 was concentrated in the presence of 2.5 mM cholate to 10–15 mg/ml using an Amicon Ultra-0.5 Ultracel-30 concentrator (Millipore) and used for reconstitution.

Reconstitution and purification of nanodiscs

Nanodisc reconstitutions were performed according to published protocols^{31,34–36} with modifications. Nanodiscs were prepared using POPC, a POPC/POPG mixture (1:1 M/M), or POPG.

For NTS1_f-nanodisc preparations, NTS1_f was concentrated to a volume of ~200 μ l using an Amicon Ultra-0.5 Ultracel-30 concentrator (14,000 rpm, 30 min, 4 °C, Eppendorf centrifuge). Lipids [solubilized in 50 mM sodium cholate, 50 mM Tris-HCl (pH 7.4), and 200 mM NaCl], ice-cold buffer A, and concentrated NTS1_f were then combined, followed by addition of concentrated MSP1D1. The final concentrations of the components were 3 mM lipids, 3–4 μ M NTS1 fusion protein, and 100 μ M MSP1D1 in a volume of 1 ml. The mixture was incubated for 1 h at 4 °C. The detergent was then removed by adding 35-fold the weight in cholate of Bio-Beads (SM-2; BioRad). After mixing for 2 h at 4 °C, the Bio-Beads were removed by centrifugation

(10,000 rpm, 3 min, 4 °C, Eppendorf centrifuge). The above procedure generated a mixture of NTS1_f-nanodiscs and nanodiscs devoid of receptor. To enrich for NTS1_f-nanodiscs, we incubated the mixture in batch for 1 h at 4 °C with 0.3 ml Talon resin (Clontech), equilibrated with buffer D. The resin was transferred into a small column and washed with 4 bed volumes of buffer D. NTS1_f-nanodiscs were eluted from the resin in 0.3-ml steps using buffer E (buffer A with 200 mM imidazole). The fractions were analyzed for protein content by measuring the absorbance at 280 nm with a NanoDrop 1000 spectrophotometer using a calculated extinction coefficient of 176,185 M^{–1} cm^{–1} and a calculated combined molecular mass of 144.3 kDa, assuming two MSP1D1 molecules and one NTS1_f molecule per nanodisc. The protein content was also determined by the method of Schaffner and Weissmann⁸⁵ with BSA as the standard. The fraction with the highest NTS1_f-nanodisc concentration was used for further experiments.

For production of empty-nanodiscs (nanodiscs without NTS1), the respective lipid/cholate solution, concentrated MSP1D1, and buffer A were mixed to give final concentrations of 5.5 mM lipid and 100 μ M MSP1D1 in a volume of 250 μ l. The mixture was incubated for 1 h at 4 °C. Bio-Beads were then added for detergent removal to 35-fold of the weight of cholate present in the mixture. After mixing for 2 h at 4 °C, the Bio-Beads were removed by centrifugation (10,000 rpm, 3 min, 4 °C, Eppendorf centrifuge).

Sedimentation velocity experiments

Sedimentation velocity experiments were conducted at 10.0 °C on a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge. Nanodisc preparations (22 μ M empty-nanodiscs, 5–6 μ M receptor-nanodiscs) were loaded in 2-channel centerpiece cells and analyzed at a rotor speed of 40 krpm. Absorbance (280 nm) and interference data were collected and analyzed in SEDFIT 12.1b.⁴⁸ Data were also analyzed, individually and globally, in SEDPHAT 8.2⁸⁶ in terms of a continuous $c(s)$ distribution of Lamm equation solutions, as well as hybrid local continuous distributions and global discrete species. In all cases, excellent fits were obtained with absorbance and interference r.m.s.d. values ranging from 0.0050 to 0.0066 A_{280} and 0.0060 to 0.011 fringes. Solution densities ρ and solution viscosities η were measured experimentally at 20.00 °C on a Mettler Toledo DE51 density meter and an Anton Paar AMVn rolling ball viscometer, respectively, and corrected to 10.0 °C. The partial specific volumes v of MSP1D1 and NTS1_f were calculated in SEDNTERP 1.09.⁸⁷ A partial specific volume of 0.979 cm³ g^{–1} was used for both POPC and POPG. This value is based on a published POPC partial specific volume of 0.9952 cm³ g^{–1} at 30.0 °C⁸⁸ and a lipid dv/dT of 0.00080–0.00083 cm³ g^{–1} K^{–1} (Refs 89 and 90). Protein extinction coefficients at 280 nm ϵ_{280} were calculated in SEDNTERP 1.09, whereas the interference signal increments ϵ_I were determined using dn/dc values of 0.185 cm³ g^{–1} for MSP1D1 and NTS1_f⁹¹ and 0.164 cm³ g^{–1} for both POPC and POPG.⁹² Sedimentation coefficients s were corrected to $s_{20,w}$ based on the expected stoichiometries (Table S1).

Dynamic light scattering

The translational diffusion coefficients D and corresponding hydrodynamic radii were measured from an

† <http://www.expasy.ch/tools/protparam.html>

autocorrelation analysis of the quasielastically scattered light at 514.5 nm. Autocorrelation functions were accumulated for 2–4 min at 19 °C and an angle θ of 90° using a Brookhaven Instruments BI-9000 AT autocorrelator with sampling times of 1.0 μ s to 5 ms. Data were imported into SEDFIT 12.1b. For NTS1_f-nanodiscs (4–9 μ M), the normalized intensity autocorrelation functions $g^{(2)}(\tau) - 1$ were used to obtain the second cumulant Γ_2 and moment μ_2 (expressed in terms of the polydispersity μ_2/Γ_2).^{93,94} In the case of empty-nanodiscs (36–47 μ M), the presence of small amounts of aggregate required an analysis of the field autocorrelation function $g^{(1)}(\tau) = \sqrt{g^{(2)}(\tau) - 1}$ in terms of two discrete species.

Lipid analysis by ¹H NMR

POPC and POPG powder was purchased from Avanti Polar Lipids. Deuterated chloroform (CDCl₃) and deuterated methanol (MeOD) were obtained from Cambridge Isotope Laboratories. To prepare the reference samples, we dissolved lipid powder (100 μ g) in CDCl₃ (600 μ l) and transferred it to a 5-mm OD Precision NMR tube. MeOD (100 μ l) was then added. The empty- and NTS1_f-nanodisc samples were first freeze-dried, then dissolved in CDCl₃, and homogenized by vortexing. Each sample was filtered through a glass wool filter into the NMR tube. The filtration step was necessary to remove chloroform-insoluble salt precipitates and invariably resulted in some small sample losses. MeOD (100 μ l) was then added to the samples. ¹H NMR experiments were performed at a resonance frequency of 500.13 MHz on a Bruker DMX500 spectrometer. Spectral assignments were made by reference to published data.⁹⁵ The integrals of the well-isolated peaks of the acyl C2 and C2' protons (2.25–2.37 ppm) were used for quantification of the total lipid amount. As the peaks specific for the head groups of POPC and POPG were not well isolated in the spectra, we did not analyze the fractional contribution of POPC and POPG in POPC/POPG-nanodiscs.

Lipid analysis by HPLC combined with tandem quadrupole mass spectrometry

The HPLC/MS/MS analyses of empty-POPC-nanodiscs and NTS1_f-POPC-nanodiscs were done by Avanti Polar Lipids (one experiment each). Briefly, nanodiscs were diluted into methanol and sonicated to help lipid dissolution. A methanol/ammonium acetate gradient was used for HPLC, followed by MS/MS optimized for POPC. The empty-nanodisc and NTS1_f-nanodisc preparations contained 4.5 ± 0.1 mg/ml (5921 μ M) POPC and 0.21 ± 0.01 mg/ml (276 μ M) POPG, respectively. The corresponding protein contents (determined by the method of Schaffner and Weissmann⁸⁵) were 1.85 mg/ml (84 μ M MSP1D1) and 0.6 mg/ml (8.3 μ M MSP1D1 and 4.16 μ M NTS1_f, assuming two MSP1D1 molecules and one NTS1_f molecule per nanodisc). These values correspond to 141 POPC molecules per empty-nanodisc and 66 POPC molecules per NTS1_f-nanodisc.

Saturation and competition agonist binding experiments

NTS1_f-nanodiscs, containing receptor with MBP at its N-terminus and a TrxA-H10 tail at its C-terminus, were

treated with Tev protease prior to ligand binding and nucleotide exchange experiments, to cleave off the tags and generate NTS1 with near-authentic N- and C-termini (Figs. 1 and 4 and Fig. S1). For this, purified NTS1_f-nanodiscs were incubated with stoichiometric amounts of Tev protease (no DTT addition) for 2 h on ice; the completeness of the digest was confirmed by SDS-PAGE.

NTS1-nanodiscs were incubated with [³H]NT and unlabeled NT on ice for 1 h in 150 μ l of assay buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 7 mM NaCl, 0.1% BSA, and 0.004% bacitracin]. Separation of the receptor-ligand complex from free ligand (100 μ l) was achieved by centrifugation-assisted gel filtration using Bio-Spin 30 Tris columns (BioRad), equilibrated with RDB buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% *n*-dodecyl- β -D-maltopyranoside, 0.2% Chaps, and 0.04% cholesteryl hemisuccinate Tris salt].

For saturation binding experiments at equilibrium, the [³H]NT concentration was varied from 0.05 nM to 12 nM. Nonspecific [³H]NT binding was determined in the presence of 50 μ M unlabeled NT. Data were analyzed by nonlinear regression using the GraphPad Prism software (version 4, GraphPad Software) and best fit to a one-site binding equation (accounting for ligand depletion) to determine the equilibrium dissociation constants (K_d). Individual experiments were conducted in duplicate.

[³H]NT at 2 nM was utilized for homologous competition experiments (NT/[³H]NT). Independent experiments were carried out in single. Ligand depletion was considered to be low (<26%). Data were best fit to a sigmoidal dose-response equation with standard slope using the concentrations of total NT added *versus* bound [³H]NT. Inhibition constant (K_i) values were derived from IC₅₀ values using the Cheng and Prusoff equation, $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of [³H]NT.⁹⁶

Single-point ligand binding experiments in the presence of 2 nM [³H]NT were conducted to monitor NTS1 during the reconstitution process. A [³³H]NT concentration of 5 nM was used to confirm the receptor density in NTS1-nanodisc preparations prior to each nucleotide exchange reaction.

GDP/GTP γ S exchange assay

The receptor-catalyzed exchange of GDP for GTP γ S on G α_q was determined as previously described^{97,98} in a total assay volume of 50 μ l. Reaction mixtures were kept on ice throughout the procedure unless indicated otherwise. NTS1-nanodiscs were added to G protein (*Sepia* G α_q and bovine G $\beta_1\gamma_1$) and agonist to give a volume of 30 μ l. GDP/GTP γ S exchange was initiated by the addition of 20 μ l of [³³S]GTP γ S mix. The final component concentrations in each sample were 50 mM Mops (pH 7.5), 1 mM EDTA, 7 mM NaCl (NT saturation) or 100 mM NaCl (G α_q and G $\beta_1\gamma_1$ saturation), 1 mM DTT, 3 mM MgSO₄, 0.3% (wt/vol) BSA, 1 μ M GDP, 4–8 nM [³³S]GTP γ S (PerkinElmer), 40 μ M adenylyl imidodiphosphate, and 0.4 mM cytidine 5'-monophosphate. Reactions were terminated by diluting the reaction mixture with 2 ml of ice-cold stop buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 25 mM MgCl₂] and were filtered over nitrocellulose membranes on a vacuum manifold. Filters were then washed six times

with 2 ml of ice-cold stop buffer. The nitrocellulose membranes were dried, and the radioactivity was quantitated by liquid scintillation in a Beckman Coulter LS 6500 scintillation counter. Note that Gαq was purified in buffer containing Chaps. However, the Gαq concentrations in the exchange assays did not exceed 1 μM (see below), thus limiting the free Chaps concentration to less than 1/10th of its critical micellar concentration.⁵⁰

Single-point individual experiments were conducted using incubation times of 120 min, 45 min, and 15 min for NTS1 in POPC-, POPC/POPG- and POPG-nanodiscs, respectively (NT saturation, Fig. 5d–f; Gαq saturation, Fig. 6a; Gβ₁γ₁ saturation, Fig. 6b). NT saturation experiments were conducted at 250 nM Gαq, 1 μM Gβ₁γ₁, 5 nM NTS1, and 0–1 μM NT. Gαq saturation experiments were done at 0–1 μM Gαq, 1 μM Gβ₁γ₁, 5 nM NTS1, and 10 μM NT. Gβ₁γ₁ saturation experiments were done at 250 nM Gαq, 0–5 μM Gβ₁γ₁, 5 nM NTS1, and 10 μM NT. The fractional contribution of the non-catalyzed nucleotide exchange in Gαq and Gβ₁γ₁ saturation experiments was estimated from reactions without NT.

For NT saturation experiments, initial rates of reaction were approximated throughout, that is, <54% of [³⁵S]GTPγS was consumed at the highest agonist concentration (Fig. 5d–f). In Gαq and Gβ₁γ₁ saturation experiments (Fig. 6), <33% and <28% of [³⁵S]GTPγS were consumed, respectively.

Data from NT saturation of NTS1-catalyzed GDP/GTPγS exchange experiments (Fig. 5d–f) were analyzed in terms of a standard sigmoidal dose–response (Hill slope=1) in GraphPad Prism. For the determination of K_m values for Gαq saturation of NT-induced, receptor-catalyzed GDP/GTPγS exchange (Fig. 6), [³⁵S]GTPγS binding in the absence of NT (i.e., the non-catalyzed nucleotide exchange at a given Gαq concentration) was subtracted from [³⁵S]GTPγS binding in the presence of NT. The resulting data, reflecting specific NT-induced nucleotide exchange, were analyzed using a one-site binding equation.

To facilitate the comparison between the three NTS1-nanodisc types (note that different incubation times were chosen for POPC-, POPC/POPG-, and POPG-nanodiscs, respectively, to allow robust nucleotide exchange but avoid [³⁵S]GTPγS depletion at an NTS1 concentration of 5 nM), we normalized the data from the Gαq, Gβ₁γ₁, and NT saturation experiments to a molar turnover number for catalyzed [³⁵S]GTPγS bound (cpm) (nM NTS1 protein)^{−1} min^{−1} by dividing the measured cpm values through the respective incubation time (min) and 5 (nM). This normalization procedure was done for the highest Gαq, Gβ₁γ₁, and NT concentrations used in the respective assays (Gαq saturation: 1 μM Gαq, 1 μM Gβ₁γ₁, 100 mM NaCl, and 10 μM NT; Gβ₁γ₁ saturation: 250 nM Gαq, 5 μM Gβ₁γ₁, 100 mM NaCl, and 10 μM NT; NT saturation: 250 nM Gαq, 1 μM Gβ₁γ₁, 7 mM NaCl, and 1 μM NT) (Table 3). Note that the affinity of NT for NTS1 is affected by the presence of Na⁺,^{99,100} reducing the efficacy of G protein activation at high NaCl concentrations. Likewise, the data in Fig. 6a and b were normalized to catalyzed [³⁵S]GTPγS bound (cpm) (nM NTS1 protein)^{−1} min^{−1} to quantitatively determine the effect of the negatively charged lipid POPG on the NTS1-catalyzed nucleotide exchange (Fig. 6c and d).

To determine whether the presence of the TrxA-H10 tag at the receptor C-terminus hinders G protein coupling to NTS1, nucleotide exchange experiments were conducted

with NTS1_F-POPC-nanodiscs in the presence (stoichiometric amounts) and absence of Tev protease (20 nM NTS1, 250 nM Gαq, 1 μM Gβ₁γ₁, ±10 μM NT, and 100 mM NaCl, 5 min at 30 °C) (Fig. 4).

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Supplementary Data

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