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Lipid modulation of early G protein-coupled receptor signalling events

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ABSTRACT

Upon binding of extracellular ligands, G protein coupled-receptors (GPCRs) initiate signalling cascades by activating heterotrimeric G proteins through direct interactions with the α subunit. While the lipid dependence of ligand binding has previously been studied for one class A GPCR, the neurotensin receptor 1 (NTS1), the role the lipid environment plays in the interaction of activated GPCRs with G proteins is less well understood. It is therefore of interest to understand the balance of lipid interactions required to support both ligand binding and G protein activation, not least since some receptors have multiple locations, and may experience different membrane environments when signalling in the plasma membrane or during endocytosis. Here, using the sensitive biophysical technique of microscale thermophoresis in conjunction with nanodisc lipid bilayer reconstitution, we show that in more native lipid environments rich in phosphatidyl ethanolamine (PE), the $G\alpha_{i1}$ subunit has a ~4-fold higher affinity for NTS1 than in the absence of native lipids. The G protein-receptor affinity was further shown to be dependent on the ligand-binding state of the receptor, with potential indication of biased signalling for the known antagonist SR142948A. $G\alpha_{i1}$ also showed preferential interaction with empty nanodiscs of native lipid mixtures rich in PE by around 2- to 4-fold over phosphatidyl choline (PC)/phosphatidyl glycerol (PG) lipid mixtures. The lipid environment may therefore play a role in creating favourable micro-environments for efficient GPCR signalling. Our approach combining nanodiscs with microscale thermophoresis will be useful in future studies to elucidate further the complexity of the GPCR interactome.

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1. Introduction

G protein-coupled receptors (GPCRs) are the largest class of cell surface receptors, and their pivotal role in cellular signalling is highlighted by the fact that they form the target for ~40% of marketed pharmaceuticals [1]. They respond to a wide array of stimuli, ranging from hormones and peptides to ions and photons. Upon ligand binding on the extracellular side of the membrane, GPCRs undergo conformational changes which lead to the activation of heterotrimeric G proteins on the intracellular side, triggering downstream signalling pathways. The cellular response depends on the G protein subtype, and specific GPCRs can couple through one or more G protein subtypes, which are typically classified by the α subunit of the heterotrimer, with four families identified to date: G_s, G_i, G_q, and G_{12}[2,3]. The Gα subunit directly interacts with the receptor through interactions with the transmembrane (TM) core (TM3, 5, and 6) and intracellular loops (IC2 and 3) [4–7], which leads to a large conformational change in the G protein allowing the exchange of GDP for GTP in the nucleotide binding pocket, and initiating downstream signalling, through the α subunit and the βγ heterodimer [4,8]. Part of the recent crystallographic success which has advanced our understanding of GPCR activation can be attributed to the use of lipidic mesophases for crystallisation [9], highlighting the importance of the lipid environment for GPCR stability. Indeed, from numerous functional studies, with a particular emphasis on the prototypical GPCR rhodopsin, it is clear that the membrane plays an important role in modulating GPCR activity (recently reviewed in [10]). However, despite often being used as a model for all other GPCRs, rhodopsin and its membrane environment present a somewhat unique case in that rhodopsin is the main protein component (>90%), and thus rod outer segment membranes (which are especially rich in PE and polyunsaturated fatty acids), will likely have evolved to support rhodopsin function (and/or *vice versa*), while the membranes in which other GPCRs function are more diverse in protein composition [11,12]. Therefore, it is of interest to test whether previous observations on the lipid preference of rhodopsin generalise to other GPCRs.

Neurotensin receptor 1 (NTS1) is a class A GPCR, expressed both in the brain and in the periphery, with pharmacological potential as target for treatment of schizophrenia, Parkinson's disease, obesity, and drug addiction, and as a biomarker for certain cancers, making it an interesting and therapeutically relevant system for functional studies [13]. Stimulation of the receptor by its natural ligand, the tridecapeptide neurotensin (NT), has been shown to lead to activation of multiple G protein subtypes: G_s, G_i, and G_q (reviewed in [14]). Mutagenesis studies have suggested that interactions with IC3 are crucial for G_q activation, while activation of G_s and G_i requires the presence of the first half of the C-terminus [15–17]. Ligand binding to NTS1 has been shown to be sensitive to its lipid environment [18]. The presence of phosphatidyl

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ethanolamine (PE) lipids was found to be specifically required, with no discernible ligand-binding activity in phosphatidyl choline (PC) lipids alone. While the presence of the cholesterol analogue CHS increased the stability of the receptor (*i.e.* increased its half-life before ligand-binding capacity is lost), CHS or cholesterol were not required for ligand binding [18]. Indeed, ligand binding to NTS1 has been observed in *Escherichia coli* membranes which lack cholesterol [19]. Porcine brain polar lipid extract (BPL, lipid headgroup composition given in Table S1) best supported receptor ligand-binding activity, demonstrating that more native lipids are indeed important for receptor ligand-binding function [18].

Here, we investigate the lipid dependence of the G protein–GPCR interaction by studying the interaction between $G\alpha_{i1}$ and NTS1 reconstituted into nanodiscs of different lipid composition using microscale thermophoresis (MST). As we previously showed for ligand binding to detergent-solubilised NTS1 [20], MST allows the determination of interaction affinities between biomolecules by optically probing the mobility of a fluorescently labelled molecule in a thermal gradient for a titration series of an unlabelled binding partner (see Fig. 1). Since the thermophoretic mobility of a molecule or complex is dependent on its size, charge and hydration shell [21], binding of the unlabelled partner to the optically probed species typically affects the thermophoretic mobility of the latter. Thus, the MST titration experiment yields a saturation binding curve reporting the observed thermophoresis (ΔF_{norm}) of the labelled species at different concentrations of the titrated unlabelled binding partner from which interaction affinities can be obtained [20]. In a novel configuration, MST measurements on wild-type, unmodified receptor were facilitated here by labelling the membrane scaffold protein (MSP) surrounding the lipid bilayer of the nanodiscs with a site-reactive fluorophore, rather than the incorporated receptor. While a previous study reported that negatively charged lipids (*i.e.* phosphatidyl glycerol lipids) are important for the G_q–NTS1 interaction [22], we show here that $G\alpha_{i1}$ has higher affinity for the receptor in more native lipid environments that are rich in PE lipids. Furthermore, we show that ligands modulate the G protein–GPCR affinity consistent with their pharmacological profile, in agreement with the cubic ternary complex model of ligand–GPCR–G protein interactions [23], and adding a new dimension to the GPCR interactome [24].

2. Methods

2.1. NTS1 expression, purification, and labelling

An additional 6-His-tag was introduced by QuickChange site-directed mutagenesis into the NTS1B construct described by White et al. [25] at the C-terminus of NTS1 before the TEV protease recognition site to create a new construct, $NTS1BH_6$, to facilitate IMAC on the cleaved receptor construct lacking the terminal fusion partners. $NTS1BH_6$ was expressed in *E. coli* and purified as described by Harding et al. [26], with some modifications. Specifically, after IMAC on solubilised lysate, the fusion partners were removed by proteolytic cleavage with TEV protease, and the receptor was further purified by ligand-affinity chromatography using N-terminally Cys-derived NT (Alta Bioscience) immobilized on Ultralink iodoacetyl resin (Pierce, Thermo Scientific) as per the instructions from the supplier. The sample was incubated with the resin for 2–3 h at 4 °C, impurities were removed by washing sequentially with 70 and 150 mM NaCl buffer (50 mM Tris–

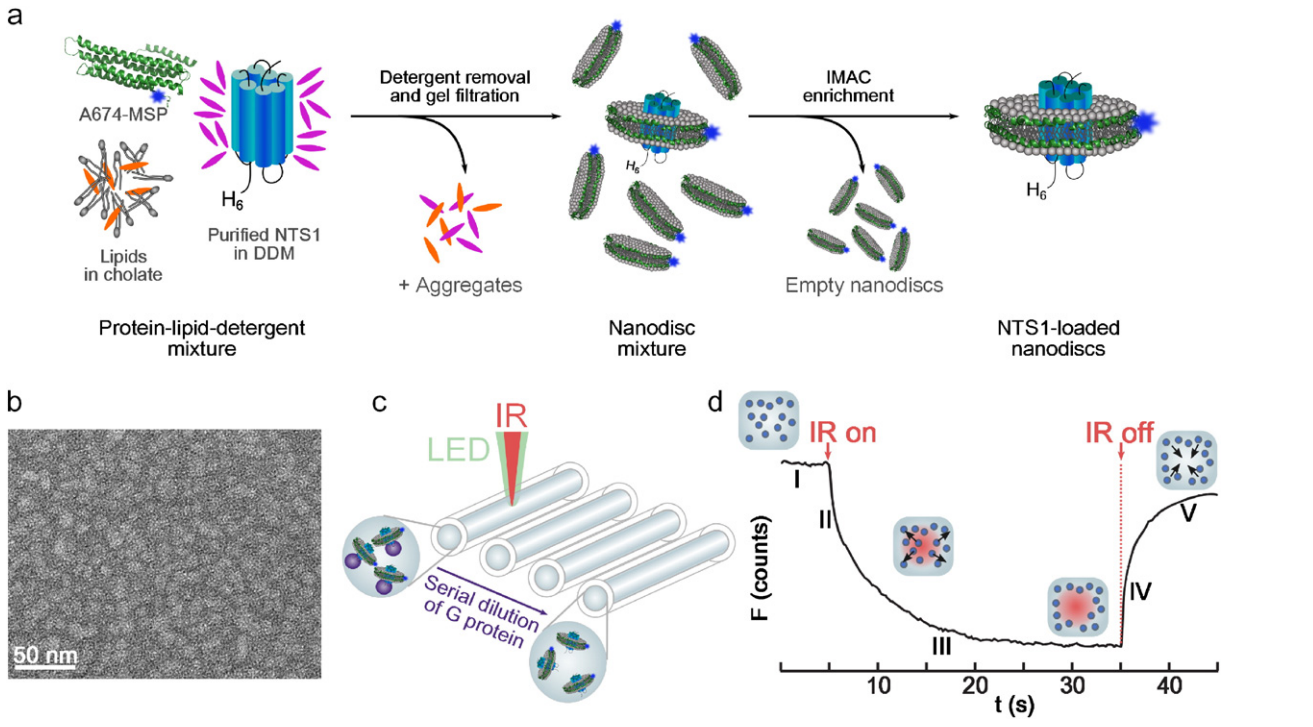

Fig. 1. MST on NTS1 nanodiscs. (a) As shown in this schematic, nanodiscs were produced by mixing lipids (grey) solubilised in sodium cholate (orange) with DDM-solubilised (pink) His-tagged (H_6) NTS1 (blue) and the MSP belt protein (green). To make fluorescent nanodiscs for MST, the MSP belt protein was labelled with an Alexa Fluor dye (A647, blue star) prior to nanodisc formation. Detergent is removed by incubation with Bio-Beads overnight, after which nanodiscs are separated from larger aggregates by gel filtration. Finally, NTS1-loaded nanodiscs are separated from empty nanodiscs by IMAC facilitated by the His-tag on NTS1. (PDB ID 3J00 and 2A01 were used for MSP.) (b) Uranyl acetate staining and transmission electron microscopy were used to image fluorophore-labelled PCPG and BPL nanodiscs (latter shown here, see also Fig. S2). (c–d) Schematic of MST setup: a dilution series of G protein (purple) is prepared to which ~5–25 nM fluorescent nanodiscs is added. Samples are loaded into capillaries, fluorophores are excited by an LED, and the initial fluorescence (F) is detected (I), after which the sample is heated locally with an IR laser (IR), giving rise to a sudden (~100 ms time scale) change in fluorescence, termed temperature jump (II). Molecules then diffuse due to thermophoresis (III). Both the T-jump and thermophoresis can be influenced by (bio)molecular interactions, and thus vary along the titration series. After the IR laser is turned off, an inverse temperature jump (IV), and back-diffusion (V) occur. The fluorescence after thermodiffusion (III) is normalised to the fluorescence before heating (I) or after T-jump (II) to yield ΔF_{norm}, which is plotted against the concentration of the titrated binding partner to obtain a binding curve [20].

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