Characterisation of a cell-free synthesised G-protein coupled receptor

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G-protein coupled receptors are the largest family of integral membrane proteins found within the human genome. They function as receptors and modulators to a wide range of ligands and responses which are crucial for human health. GPCR study, specifically the investigation of structure and interaction to cognate ligands, is of high priority. Limitations for structural study can be traced in part, to obtaining suitable quantities of recombinant protein. We sought to address the limitations of traditional recombinant technologies by utilising an Escherichia coli based cell-free protein synthesis (CFPS) approach for production of a thermostable neurotensin receptor 1 (en2NTS1). Initial results were promising, with a high amount (up to 2 mg/mL) of en2NTS1 produced, that had attained correct secondary structure. Meanwhile, concurrent experiments indicated that CFPS produced en2NTS1 showed non-competitive binding to the peptide ligand neurotensin8–13 when compared to E. coli produced en2NTS1. 1H-13C HMQC SOFAST NMR spectra were indicative of disrupted tertiary structure for CFPS produced 13CH3-methionine labelled en2NTS1. The results obtained, indicate CFPS produced en2NTS1 is not forming a discrete tertiary structure and that further development of the CFPS technique needs to be carried out.

G-protein coupled receptors (GPCRs) are a large family of integral membrane proteins of approximately 800 members in humans1,2. They constitute the largest group of cell-surface proteins in the human genome and are sorted into four families based on characteristic protein sequences and structural similarity to their prototypical namesake: the rhodopsin family (Class-A), the secretin and adhesion family (Class-B), the glutamate family (Class-C) and the Frizzled/Smoothened family (Class-F)1,3. As membrane proteins, GPCRs are the target of many different stimuli that affect a diverse range of responses. Many GPCRs are known to have broad effects on health4, and therefore, the large number of GPCRs in the human genome may allude to high numbers of possible pharmacological targets for treatment of many ailments. Notably, it has been estimated that 30–40% of all currently marketed pharmaceuticals directly target GPCRs5. As a result, the investigation of GPCR structure and function is of high importance.

The characteristic feature shared between all GPCRs is that they possess a transmembrane (TM) domain composed of seven predominantly α-helical transmembrane passes (7TM)2. The 7TM helices are arranged in a serpentine pattern in relation to the lipid bilayer and all GPCRs exhibit the same orientation with respect to their insertion into the lipid bilayer: the N-terminus is exposed to the extracellular face of the lipid bilayer and the C-terminal tail projecting into the intracellular space. The individual TM helices are connected by a series of extracellular and intracellular loops. To date, there have been greater than 115 different GPCR structures, of varying conformational states, deposited into the PDB. Most of the successfully obtained GPCR structures have been determined from protein that is produced by recombinant means. The most common expression organisms used for production of GPCRs include insect cells, Spodoptera frugiperda or Trichopulsia ni and the yeast, Pichia pastoris. The main attribute for expression of GPCRs using these organisms, is that they are processed through a similar secretory pathway as in mammalian hosts. Specifically they allow facilitation of receptor folding pathways,

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protein processing and disulphide bond formation. Additionally, post-translational modifications (PTMs) such as glycosylation may occur, albeit differing in form depending on expression host.

Alternative production techniques for GPCRs include *Escherichia coli* expression or cell-free protein synthesis (CFPS). Expression in *E. coli* is a proven method for expression of some native or mutant GPCRs in a properly folded form that exhibit native ligand binding. Examples include A2AR, β1AR, CXCR1 and NTS1. Meanwhile CFPS of folded and functionally active GPCRs has only been successful on a few receptors such as the β2AR, H1R and dopamine D2R. Despite limited success, CFPS is a highly sought after technology to produce GPCRs due to the relatively low cost of materials and high yields of protein production. Additionally, CFPS allows the user to directly manipulate and also include unnatural components to the synthesis reaction. The substitution of isotopically labelled or unnatural amino acids makes CFPS a very attractive alternative to traditional recombinant expression. However, expression of membrane proteins by CFPS is challenging, as expression is carried out with minimal or negligible regulation of protein folding. Therefore the operator must supply an environment suitable for solubilisation of the nascent protein or include a refolding step following expression.

Described here is the investigation to assess the functionality and structure of an engineered thermostabilised neurotensin receptor 1 (en2NTS1) produced by CFPS. The work presented demonstrates the capability of CFPS to produce high levels of GPCR protein. The system allowed significant manipulation, by addition of various detergents and replacement of unlabelled methionine with 13C-methyl-methionine for eventual studies of GPCR structure and dynamics by NMR spectroscopy. However ligand binding assays revealed that CFPS produced malE-en2NTS1 showed a propensity to bind non-competitively to the peptide ligand neurotensin8–13 (NT8–13). Additionally, NMR studies highlighted that CFPS produced en2NTS1 displayed a non-native tertiary structure, compared to *E. coli* produced en2NTS1, consistent with CFPS producing a poorly folded, non-functional receptor.

Results

en2NTS1 cloning. The first requirement for establishing CFPS production of en2NTS1 was constructing the necessary template for protein synthesis. Based on previous reports which state that reducing mRNA fold at the 5′-end of coding mRNA can lead to optimal likelihood of translation initiation and protein expression, we sought to create several N-terminally tagged-based en2NTS1 variants. Creation of the tagged-en2NTS1 variants utilised an overlap PCR approach (Fig. 1A) (Fig. S1 in Supporting Information), whereby nine fusion partners were chosen based on their reported expression enhancement of both soluble and membrane proteins by CFPS. The impact of the different expression tags on protein synthesis was performed by analytical scale CFPS expression in the absence of surfactants (precipitate mode CFPS: P-CFPS) so as to allow easy isolation of
the product by centrifugation. Expression was observed for most of the N-terminal tags tested, despite an overall lower expression for the linear templates compared to an intact plasmid. Despite the low expression, two signal sequence based tags obtained from malE and ompC promoted sufficient expression, with the 26-residue malE tag producing approximately 47% more product than ompC tagged en2NTS1 (Fig. 1B). Thus malE-en2NTS1 cloned into pETMCSI (a kind gift from K. Ozawa and G. Otting) was therefore used for continued study.

**malE-en2NTS1 expression optimisation and purification.** Further optimisation of malE-en2NTS1 expression was carried out by testing varying Mg$^{2+}$ concentrations, expression in the presence and absence of detergent and determining the relative yield of protein from CFPS. Titration with Mg$^{2+}$ showed that optimal expression could be obtained at a concentration of 17 mM. Expression could be carried out in the presence of Brij35 (0.1% w/v) and Brij58 (1.5% w/v), with noticeably better expression in Brij58 because a large proportion of malE-en2NTS1 was insoluble with Brij35 addition (Fig. 2A). Notably, addition of Triton X-100, dodecylphosphocholine (DPC), decyl maltoside (DM) or dodecyl maltoside (DDM), resulted in no malE-en2NTS1 expression. A comparison between detergent mode-CFPS (D-CFPS) using Brij58 and P-CFPS showed P-CFPS generated higher levels of synthesised protein. Overall yields by D-CFPS and P-CFPS were 0.5–1 mg mL$^{-1}$ and 1.5–2 mg mL$^{-1}$ respectively. For malE-en2NTS1 expressed in the P-CFPS mode, complete solubilisation of the precipitate was successfully carried out using 21 mM (1% w/v) 1-myristoyl-2-hydroxy-sn-glycero-3-phospho-(1′-rac-glycerol) (LMPG) in nickel equilibration buffer.

Purification utilised Ni-IMAC to produce a product that was ≥90% pure for both D-CFPS and P-CFPS derived malE-en2NTS1 (Fig. 2B,C respectively). All samples were purified in either Brij58 (D-CFPS derived), LMPG (P-CFPS derived) or DDM (buffer exchanged from D-CFPS or P-CFPS derived malE-en2NTS1). Unfortunately malE-en2NTS1 was unable to be detergent exchanged into DM. Several attempts were carried out, however all attempts resulted in precipitation of malE-en2NTS1. The precipitated material was determined to be...
malE-en2NTS1 by SDS-PAGE (data not shown). For malE-en2NTS1 purified in either DDM, LMPG or Brij58, size-exclusion profiles show monodisperse profiles (Fig. 2D). The varying elution volumes were predicted to be due to the different micellar properties of the solubilising detergents (Fig. S2 in Supporting Information).

Circular Dichroism and secondary structure. Circular dichroism (CD) was carried out to determine the estimated secondary structure of malE-en2NTS1, and E. coli produced en2NTS1. The samples used for CD were derived either from D-CFPS and P-CFPS expressed malE-en2NTS1. In both cases however, detergent exchange to DDM took place during the Ni-IMAC step and DDM was maintained during subsequent SEC and CD steps. The far-UV wavelength CD profile of malE-en2NTS1, which includes the 26-residue malE tag, displayed a content of secondary structure consistent with that of a folded GPCR (Fig. 3A). This was broadly similar to the control CD profile obtained for E. coli produced en2NTS1, however the mean residue ellipticity is lower for the cell-free produced material (Fig. 3B). The two double minima present at 208 and 222 nm were suggestive of a protein with a high propensity of \( \alpha \)-helix. The reconstituted data were fitted best with the algorithms CDSSTR and CONTILL, producing the lowest nRMSD values and therefore a more accurate fit of the acquired data\(^9\) (Table 1). When analysed, the overall secondary structure composition calculated by CDpro produced an estimation of \( \alpha \)-helix similar to data obtained from a second program, STRIDE\(^20\). The calculated \( \alpha \)-helical percentage (58–60%) and the limited amount of \( \beta \)-strand (8–13%) was similar to the calculated CD profile for E. coli produced en2NTS1, as well as the amount determined for the NTS1-OGG7 crystal structure (OGG7-ΔIC3, PDB: 4BV0), to which both malE-en2NTS1 and en2NTS1 share many of the same stabilising mutations\(^10\) (Fig. S3 in Supporting Information). This analysis is suggestive of malE-en2NTS1 assuming a structure that is consistent with correct secondary fold, however the lower value of the mean residue ellipticity is consistent with poor packing of the \( \alpha \)-helices to form the correct tertiary structure\(^21\). Clearly, in the absence of the E. coli expressed en2NTS1 such an analysis is not possible.
Streptavidin and His6-tag pulldown assays of malE-en2NTS1. Automated streptavidin binding experiments were carried out using the Kingfisher particle processor. Streptavidin-coated dynabeads were used to capture biotinylated NT, which was subsequently used to capture CFPS produced malE-en2NTS1 (Fig. 4A). *E. coli* expressed en2NTS1 (Fig. S3) served as a control for expected interaction of the receptors with the NT8–13 ligand (Fig. 4B). The experimental set up included three conditions: 1: Receptor alone, 2: biotinylated-NT and receptor and 3: biotinylated-NT, receptor and unlabelled NT8–13 competitor. No binding of malE-en2NTS1 or of en2NTS1 was detected to the streptavidin dynabeads in the absence of bound biotin-NT. Addition of biotin-NT promoted binding of malE-en2NTS1 and en2NTS1 to the beads. Addition of competing unlabelled NT8–13 caused a reduced level of bound en2NTS1, but did not change the level of bound malE-en2NTS1 to the biotin-NT-coated streptavidin dynabeads. For malE-en2NTS1, samples obtained following Ni-IMAC in the detergents Brij58, LMPG or DDM produced the same result, as did samples purified by SEC in DDM (data not shown).

In light of the streptavidin pulldown experiments, an alternative approach was attempted. malE-en2NTS1 was designed with a His10-tag at its C-terminus, a trait that is exploited during its purification. With this in mind, the His-tag was used to reverse the original streptavidin method for detection of possible binding of NT8–13. By coupling the malE-en2NTS1 receptor to His-tag isolation dynabeads, detection of binding can be made through Alexa647 fluorescently labelled NT14. Once again, malE-en2NTS1 binding experiments could be compared in a similar manner to the *E. coli* expressed en2NTS1 control which can also be captured via its His-tag. Results from several experiments however produced similar results to the previous streptavidin assay (Fig. 4C,D). In the absence of bound receptor, A647-NT8–13 showed no interaction with the His-tag isolation dynabeads. Binding of both receptors to the His-tag isolation dynabeads, promoted binding of A647-NT8–13. The co-incubation of excess unlabelled NT8–13 as a competitor resulted in no dissociation of A647-NT8–13 from CFPS produced malE-en2NTS1, whereas complete competition of A647-NT8–13 binding was observed from *E. coli* produced en2NTS1 immobilised beads (Fig. 4D).

1H-13C SOFAST HMQC NMR of en2NTS1. 13CH3-methionine labelled malE-en2NTS1 was purified from either P-CFPS or D-CFPS derived sources in DDM and Brij58 detergents respectively. malE-en2NTS1 in the NT agonist bound state was recorded by adding excess NT8–13 in a ratio of 10:2 NT8–13:receptor (100 μM:20 μM). Following NMR acquisition, the sample was checked for any precipitation by visual inspection of the sample. Spectra were obtained for malE-en2NTS1 (apo and 100 μM NT8–13) in the presence of 1 mM DDM or 1 mM Brij58.

MalE-en2NTS1 has three methionines in the malE tag and nine within the transmembrane domain (Figs S3 and S4 in Supporting Information). However, the spectra for apo malE-en2NTS1 in both detergents displayed two broad signals (Fig. 5A,C) compared to *E. coli* derived en2NTS1 in DDM, which showed clearly defined resonances.
for at least six of nine possible methionine signals (Fig. 5F). Alternatively, addition of NT8–13 to CFPS-derived malE-en2NTS1 promoted no change in the size of the resonance, nor was there any noticeable change in chemical shift (Fig. 5B,D) whereas addition of NT8–13 to E. coli derived en2NTS1 resulted in chemical shift and/or line width changes for four of the resonances (Fig. 5G). Of particular note was the observed shape of both P-CFPS and D-CFPS derived forms of malE-en2NTS1, where a comparison of resonances derived from samples purified from D-CFPS and P-CFPS malE-en2NTS1 (Fig. 5A,C) indicate, that regardless of the CFPS method for malE-en2NTS1 production, the final purified forms are displaying similar properties.

MalE-en2NTS1 labelled with 13CH3-methionine was expressed in the presence of POPC nanodiscs. NMR was carried out on Ni-IMAC purified malE-en2NTS1, in the same manner used for detergent purified malE-en2NTS1. From the spectra, only a large single resonance was detectable, although the line-width of this single resonance compared to DDM/Brij58 solubilised malE-en2NTS1 appeared narrower (Fig. 5E). Comparison to the spectra obtained for en2NTS1 (Fig. 5F) show none of the defined resonances expected of a protein adopting a distinct tertiary structure.

Following NMR acquisition, the sample was applied to SEC to identify whether the nanodisc fraction still maintained the expected elution profile. Nanodiscs that utilise the MSP1D1 scaffold have a defined diameter of ~100 Å or 10 nm without embedded membrane protein and may be slightly larger with protein embedded22, 23. When compared to protein standards, nanodiscs of correct size eluted at the same volume as catalase, due to their shared Stokes diameter. For the malE-en2NTS1/POPC complex, the elution did not overlap with the expected elution for catalase, instead eluting in the predetermined void-volume, which is indicative of misfolded protein (Fig. S5 in Supporting Information). From the SDS-PAGE of the fractions obtained by SEC, it can be concluded that the protein was associated with the MSP1D1 scaffold, however the “complexes” were very large, suggesting that malE-en2NTS1 had possibly promoted disruption of the disc structure.

It should be noted here, that we have made attempts to incorporate purified malE-en2NTS1 into nanodiscs and liposomes composed of POPC, DMPC or E. coli total lipid extract. Regrettably all attempts to reconstitute malE-en2NTS1 resulted in protein destabilisation and protein precipitation following detergent removal by use of biobeads or dialysis. For nanodisc reconstitution, changing the incubation time, ratios of malE-en2NTS1, lipid and MSP1D1 scaffold protein did not prevent protein precipitation.

Discussion

G-protein coupled receptors are a diverse set of membrane proteins that respond to an equally diverse range of external stimuli. The many roles that GPCRs play in human physiology highlights their importance and therefore their continued study. Over the years, techniques to produce GPCRs recombinantly have led to an expansion in the details surrounding their structure and function. GPCRs have typically been generated via insect (S. frugiperda or T. ni) or yeast (P. pastoris) cell expression, which allows for processing by the secretory pathway24, 25. Alternate expression methodologies include that of E. coli expression, by both membrane integration26, 27 or by inclusion body formation28. Cell-free protein synthesis offers a fourth alternative for the production of GPCRs29–31, with exciting prospects for introducing unnatural amino acids and for allowing direct access to solubilising detergent, all of which formed the basis for this work.

Outlined here is the design, production, purification and initial characterisation of cell-free produced malE-en2NTS1, a thermostable NTS1 receptor14. This receptor was chosen for its proven ability for expression in E. coli cell systems (the basis for the CFPS extract used here), high stability in short chained detergents and at elevated temperature and more recently a similar variant was successfully crystallised and its structure determined32.

Work proceeded first with en2NTS1 by designing suitable N-terminal tags for the quick generation of PCR templates. This methodology allows for the production of multiple templates for rapid determination of expression16–18, 28, 29. Nine tags were chosen with PCR templates for all tags generated in one day. CFPS proceeded the following day and determination of protein expression on the third day. Ultimately CFPS of the malE-tagged
en2NTS₁ demonstrated that it was the optimal construct, with high levels of protein production in the 1.5–2 mg/mL range.

Introduction of individual detergents into the CFPS reactions was shown to promote or perturb malE-en2NTS₁ solubility. Of the detergents tested, only Brij35 and Brij58 resulted in soluble protein expression, with Brij58 promoting complete solubilisation (Fig. 2A). Alternatively samples of malE-en2NTS₁ derived from CFPS reactions in the P-CFPS mode were able to be solubilised into a refolding solution containing 1% (w/v) LMPG. The properties of malE-en2NTS₁ detergent solubilisation, during D-CFPS and following P-CFPS, show consistencies with previously published work[10]. Surprisingly however, problems arose during detergent exchange steps, where either Brij58 or LMPG were exchanged to DM. In all instances of detergent exchange to DM, protein destabilisation was observed, culminating in overt protein precipitation. Comparatively, detergent exchange into DDM resulted in no precipitation. malE-en2NTS₁ desolubilisation in DM was not expected, considering the reported stability of en2NTS₁, in shorter and thus more destabilising detergents, such as octyl-glucoside (OG)[10,14]. However, the observed differences in stability were noted to possibly be in part due to the slight differences in protein sequence between malE-en2NTS₁ and en2NTS₁, such as the addition of the malE-tag.

Investigation of secondary structure by CD showed that malE-en2NTS₁ possessed the expected α-helical elements of a folded GPCR and matched the estimated secondary structure elements of the E. coli produced en2NTS1 and the solved NTS1-OGG7 structure. However, despite the optimistic structural characteristics of elements of a folded GPCR and matched the estimated secondary structure elements, it possibly had not adopted a suitably discrete tertiary structure, which does not increase the propensity to adopt the correct fold, unassisted, from an unfolded/semi-folded state. If this is the case, then refolding of malE-en2NTS₁ by employing a mild LMPG solubilisation or directly into a detergent may not be sufficient to reverse any structure assumed during in vitro translation.

It is also possible that thermal stability of en2NTS₁ gained through successive rounds of directed evolution does not increase the propensity to adopt the correct fold, unassisted, from an unfolded/semi-folded state. Likewise, malE-en2NTS₁ derived from CFPS did not respond in a manner expected of a functional receptor. While most native GPCRs would unfold in conditions where short chain detergents are present, en2NTS₁, produced in E. coli does not. Thus, the structural stability of bacterially produced en2NTS₁, in detergents such as DDM, DM or OG is contingent on the receptor first being properly folded in the E. coli membrane environment conducive to receptor folding, prior to detergent solubilisation.

One approach to supplying nascent malE-en2NTS₁, with a lipid bilayer was undertaken by supplementing the CFPS reaction with preformed nanodiscs[35] comprised of a POPC core and MSP1D1 scaffolding. Recently there have been several reports of direct CF expression of GPCRs into lipid nanodiscs[34,35]. These embedded receptors were also reported to exhibit activity, therefore offering a new method for obtaining this class of receptor in a biologically active form. Successful CFPS expression of malE-en2NTS₁ in the presence of nanodiscs and their subsequent purification also hinted at the possibility of this receptor being embedded within the nanodisc lipid core. As before, the ¹H-¹³C HMQC SOFAST acquisition was unable to detect individual dispersed resonances exhibited by E. coli produced en2NTS₁. Unfortunately, a comparison of malE-en2NTS₁ associated with the POPC nanodiscs showed a single resonance dispersal pattern similar to that of detergent solubilised malE-en2NTS₁. Following NMR acquisition, the malE-en2NTS₁/nanodisc sample was applied to gel filtration, which confirmed the aberrant NMR results were most likely due to misfold and collapse of the preformed nanodisc structure.

While the goals we set out to achieve were not met, a number of useful procedures could be implemented as generic means for assessing membrane proteins produced by CFPS. Notably the use of competition binding assays to identify whether interactions with target substrates/ligands are correct. Alternatively, if a suitable ligand is unavailable, isotopic labelling during CFPS, such as ¹³CH₃-methionine, can be used for an economic and rapid determination of protein fold.

For malE-en2NTS₁, it seems likely that it would not be possible to obtain a functional form of this receptor when derived from CFPS, unless a more radical approach to its production is undertaken. This would require malE-en2NTS₁ expression to be undertaken in the presence of a fully functional membrane translocon. This has been trialled before with microsomes or through trials of membrane incorporation with components of SecYEG and affiliated proteins[46]. In another test case, GPCRs have been incorporated into giant unilamellar vesicles originating from endoplasmic reticulum[37]. Alternatively, incorporation of E. coli microsomes derived from inner membrane vesicles (IMV) could possibly serve as a substitute to ER derived membranes. One study has shown E. coli IMVs are capable of incorporating two E. coli transporters, mannitol permease (MtlA) and the tetracycline pump (TetA)[38]. Of the total protein expressed, MtlA and TetA were successfully incorporated into the microsome bilayer at 38% (130µg/mL) and 66% (570µg/mL) respectively[38]. The yields for CFPS membrane incorporation are...
Methods
enNTS1 plasmid construction. PCR was used to generate several different linear fused-gene DNA templates of enNTS1, each with their own unique expression tag for quick determination of levels of expression by CFPS (Fig. S1 in Supporting Information). The constructs were designed to have en2NTS1 preceded by an N-terminal expression tag followed by a 3C-protease site and succeeded by a C-terminal His10-tag. The following expression tags were selected based on previously reported accounts: the signal sequences of E. coli ompA, ompC, malE38, an HA-tag (Roche pIVEX2.6d vector), truncated T7-tag (MASMTG)39, and the systematically designed expression tags AT, SER, H and G37. Once determination of optimal en2NTS1 expression was made, the thermo-stabilised en2NTS1 gene was subcloned using the Ndel and EcoRI restriction sites into pETMCSI. Generation of the malE-en2NTS1 fusion, was generated by a combination of overlap PCR, using the primers P1, P8, P9, P14 and P15 (Table S1 in Supporting Information).

Continuous exchange cell-free protein synthesis. Preparation of E. coli BL21(DE3) S30 extracts as well as the CFPS reaction was performed as described by Apponyi et al.39 with minor changes described below. E. coli S30 extract production was performed using the BL21(DE3) Rosetta strain. These latter cells were grown using the YTPG growth medium described in Choi et al.40. The cell culture OD600 was monitored during the growth phase. Once the OD600 reached 0.85, the culture was induced with 1 mM IPTG allowing production of T7RNAP to take place, thus preventing separate expression and purification of this vital component.

Each CFPS reaction was composed of 0.8 mM rNTPs (CTP, UTP, GTP), 1.2 mM ATP, 55 mM HEPES pH 7.2, 68 μM Folic acid, 0.64 mM 3′,5′-cyclic AMP, 1 mM DTT, 27.5 mM Ammonium acetate, 1 mM amino acids, 80 mM creatine phosphate, 290 mM Potassium acetate, 16–17 mM Magnesium acetate, 7.7 mM Sodium azide, 1x protease inhibitor, 1 mM six amino acid mix (RCWMD)36, 0.3 U RNase inhibitor, 250 μg mL−1 β-galactosidase, 175 μg mL−1 E. coli total tRNA, 20–40% (v/v) S30 extract, 16 μg mL−1 DNA plasmid template. For reactions requiring use of 13C14-methionine, amino acid stocks incorporated 0.5 mM 13C14-methionine and 1 mM of the 19 other amino acids.

For continuous exchange CFPS the reaction chamber was prepared as described in Apponyi et al.39. The reaction chamber was separated from a feeder chamber which supplies new substrate for continued protein production. This was achieved by enclosing the contents of the reaction chamber (S30 extract, plasmid DNA, RNase inhibitor, creatine kinase and E. coli tRNA) in dialysis tubing. The dialysis bag is then placed in a feeder solution which supplies new substrate and also serves to minimise undesirable products from the protein synthesis reaction. Reactions were expressed in a 1:14 volume ratio (reaction chamber: feeder chamber volume) and incubated at 30 °C with orbital shaking at 160 rpm for 16 hours. Protein expression was carried out in either the presence or absence of detergent or nanodiscs; D-CFPS, P-CFPS or nanodisc (ND-CFPS) modes respectively.

Nanodisc production. Nanodisc production and assembly was performed with the MSP1D1 construct according to previously established protocols32. For this work, POPC nanodiscs with a diameter of ~100 Å were produced and utilised during CFPS at a concentration of 50 μM.

Protein purification of protein from cell-free synthesis. Precipitate mode CFPS – protein solubilisation. Precipitated malE-en2NTS1, produced by P-CFPS was first separated from the overnight reaction mix by centrifugation at 17,000 × g for 10 minutes. The isolated malE-en2NTS1 required an initial detergent solubilisation step in 2 mM LMPG in nickel equilibration buffer (20 mM NaHPO4-NaH2PO4 pH 7.5, 500 mM NaCl, 5 mM imidazole pH 7.5). The CFPS produced malE-en2NTS1 was allowed to mix for one hour with shaking at room temperature. Once solubilisation was complete, the solution was clarified by centrifugation at 30,000 × g for 10 minutes. The clarified solution was then used for Ni-immobilised metal affinity chromatography (Ni-IMAC).

Detergent mode CFPS. Following overnight D-CFPS of malE-en2NTS1, the final reaction required solution clarification by centrifugation at 17,000 × g for 10 minutes. The sample solution was then adjusted to match the nickel equilibration buffer by dilution whilst maintaining the minimum CMC of the utilised detergent. This final solution was then used for Ni-IMAC.

Chromatography purification. malE-en2NTS1 samples were incubated with 2 mL of prequillified Ni-sepharose (GE Healthcare) for 1 hour at 4 °C. After collecting the flow through, the Ni-sepharose was washed with 10 column volumes of buffer (20 mM NaHPO4-NaH2PO4 pH 7.5, 500 mM NaCl, 5 mM imidazole pH 7.5, and appropriate detergent). If detergent exchange into DDM (20 mM or 1% (w/v)) was to be incorporated, this would occur during the wash step. Protein was eluted using 5 column volumes of elution buffer (20 mM NaHPO4-NaH2PO4 pH 7.5, 500 mM NaCl, 500 mM imidazole pH 7.5, and appropriate detergent). Detergent concentrations were dependent on the individual CMC of the detergent used.

Ni-IMAC purified malE-en2NTS1 was loaded onto a Superdex200 10/300 GL column pre-equilibrated with 20 mM NaHPO4-NaH2PO4 pH 7.5, 150 mM NaCl and appropriate detergent. The size exclusion step was carried out using an FPLC system (Åkta basic, GE healthcare) with buffer kept at 4 °C at a rate of 0.4 mL/min and detected by absorbance at 280 nm. Fractions of importance were collected and analysed by SDS-PAGE for purity.

en2NTS1 expression and purification from Escherichia coli. Cloning and expression. The en2NTS1 sequence was subcloned into a pQE-30-derived vector. The open reading frame of the modified vector encoded currently medium-low for these types of systems, owing to limitations in incorporation rates and total membrane area. Whether non-E. coli derived proteins could be incorporated into an E. coli based CFPS system with E. coli derived IMVs, is a matter for future investigation. However this may offer a potential method for production of folded and functional in vitro synthesised membrane protein and GPCRs as a whole.
an N-terminal maltose-binding protein signal sequence (MBPss), followed by a His\textsubscript{10}-tag, a maltose binding protein (MBP), a NNNNNNNNNNGG linker and a HRV 3 C protease site which were linked via a BamHI restriction site (resulting in additional residues GS) to residue T42 of the receptor. C-terminally T420 of the receptor was linked via a Nhel restriction site (resulting in additional residues AS) to an Avi-tag for in vivo biotinylation, a HRV 3 C protease site, a GGSGGS linker and a monomeric ultra-stable green fluorescent protein (muGSFP)\textsuperscript{49}, which is followed by a second His\textsubscript{10}-tag.

Expression and \textsuperscript{13}CH\textsubscript{3}-methionine labelling of the MBP-en2NTS\textsubscript{1}-musGFP fusion protein was carried out in \textit{E. coli} C43(DE3) (Lucigen) following an adapted protocol described by Van Duyn et al.\textsuperscript{44}.

Chromatography purification. Cell pellets were resuspended in 100 mM HEPES, 400 mM NaCl, 20% Glycerol, pH 8 with 1x EDTA free protease inhibitor tablet (Roche), 100 mg Lysozyme, 10 mg DNase and sonicated on ice. Following sonication 15 mL of DM solution (1.6 g n-decyl-β-D-maltopyranoside, Anagrade (Anatrace) dissolved overnight in water) and 15 mL of CHS/CHAPS solution (0.12 g cholesterol hemi succinate (Sigma) and 0.6 g CHAPS-hydrate (Sigma) dissolved overnight in water) were added and the volume was adjusted to 100 mL. The solubilisation mix was left gently rocking for 2 h at 4 °C. Cell debris was removed by centrifugation.

Filtered (45 μm) supernatant was adjusted to 10 mM imidazole, passed over 6 mL of Talon resin, washed with 2 x 25 mL of 25 mM HEPES, 500 mM NaCl, 10% Glycerol, 0.15% DM, 10 mM Imidazole, 0.2 mM PMSE, 8 mM ATP, 10 mM MgCl\textsubscript{2}, pH 8. Detergent was exchanged to DDM (n-decyl-β-D-maltopyranoside) (Anagrade, Anatrace) by washing with 2 x 25 mL of 25 mM HEPES, 100 mM NaCl, 10% Glycerol, 0.05% DDM, 0.2 mM PMSE, pH 8. The fusion protein was eluted using 15 x 1 mL of 25 mM HEPES, 100 mM NaCl, 10% Glycerol, 0.05% DDM, 350 mM Imidazole, 0.2 mM PMSE, pH 8.

The eluate was concentrated to 1 mL using an Amicon Ultra 15 concentrator with 100 kDa cutoff (Millipore); buffer exchanged using a PD10 desalting column (GE Healthcare) into 25 mM HEPES, 300 mM NaCl, 10% Glycerol, 0.05% DDM, pH 8. Proteolytic cleavage was carried out by adding 100 mM of Na\textsubscript{2}SO\textsubscript{4}, 1 mM TCEP and 30 μL of GST-tagged HRV 3C protease (96 μM stock produced in house) to the 4 mL PD10 eluate and rocking gently for 16 h at 4 °C. The protease was removed by mixing for 1 h at 4 °C with 2 mL of Glutathione Sepharose 4B (GE Healthcare). The flow-through was collected and the GST resin washed with 15 mL of buffer. GST flow-through and wash were combined, adjusted to 5 mM Imidazole and transferred to a gravity flow column containing 6 mL of Talon resin equilibrated with 2 x 25 mL of 25 mM HEPES, 300 mM NaCl, 10% Glycerol, 0.05% DDM, pH 8. The mixture was rocked for 45 min at 4 °C. The flow-through containing cleaved enNTS\textsubscript{1} was collected, the beads washed with 3 x 10 mL of buffer.

The flow-through and washes were combined and concentrated to 450 μL using an Amicon Ultra 15 concentrator with 30 kDa cutoff (Millipore). The concentrate was transferred to an Eppendorf tube and centrifuged in a table-top centrifuge (10000 rpm, 4 °C, 10 min) to separate any aggregated protein. The supernatant was loaded and further purified on a Superdex 200 10/300 Increase column (GE Healthcare) equilibrated with 50 mM potassium phosphate, 100 mM NaCl, 0.02% DDM, pH 7.4.

Circular dichroism spectroscopy. Far UV circular dichroism (CD) spectroscopy was carried out to estimate the secondary structure of malE-en2NTS\textsubscript{1}. Measurements were performed on an AVIV Model 410 SF spectropolarimeter (Aviv) equipped with a temperature controlled jacket set at 25 °C. malE-en2NTS\textsubscript{1} samples were maintained in a minimal salt buffer (20 mM Na\textsubscript{2}HPO\textsubscript{4}-NaH\textsubscript{2}PO\textsubscript{4} pH 7.5, 50 mM NaCl, 0.3 mM DDM). Wavelength scans were assayed at the far-UV range from 250 to 190 nm at 0.5 nm intervals and in triplicate for averaging. Protein samples (0.1 mg mL\textsuperscript{-1}) were housed in a 1-mm light path quartz cuvette (Starna). Data measured from wavelengths 250–190 nm were expressed as mean residue ellipticity [\textmu\textdegree] and fitted using the CDpro software\textsuperscript{45} running three algorithms to provide an estimate of the secondary structure composition; SELCON3\textsuperscript{46}, CDSSTR\textsuperscript{47} and CONTINLL\textsuperscript{48}. All algorithms used the protein reference set SMP56\textsuperscript{49} which includes 43 soluble proteins and 13 membrane proteins for comparison.

Ligand binding assay. Ligand binding assays of malE-en2NTS\textsubscript{1} and en2NTS\textsubscript{1} were performed on the KingFisher Flex Magnetic Particle Processor (Thermo Scientific) carried out at 4 °C. For malE-en2NTS\textsubscript{1} pulldown experiments, two different dynabead matrices were used; Streptavidin coupled M-280 dynabeads or His-tag isolation and pulldown dynabeads (Life Technologies). Streptavidin coupled M-280 dynabeads were incubated with biotinylated neurotensin (produced in house via reaction between maleimide-PEG2-Biotin and a cysteine modified body (1:1000, Cell Signalling Technologies) followed by binding of anti-mouse-Alexa488 antibody (1:5000, Life Technologies). Competition was assessed for fluorescence of Alexa488 (495 nm excitation and emission at 519 nm) measured on a POLARstar Omega plate reader (BMG Labtech). Alternatively His-tag isolation and pulldown dynabeads were used to capture malE-en2NTS\textsubscript{1} or en2NTS\textsubscript{1} for one hour. Ligand binding was assessed through binding of the Alexa647 labelled NT\textsubscript{8-13} and competition assessed by introducing unlabelled NT\textsubscript{8-13} Alexa647 fluorescence (650 nm excitation and emission at 668 nm) were also measured on a POLARstar OMEGA plate reader.

NMR Spectroscopy. Purified \textsuperscript{13}CH\textsubscript{3}-Methionine labelled malE-en2NTS\textsubscript{1} or en2NTS\textsubscript{1} was exchanged in NMR buffer (20 mM Na\textsubscript{2}HPO\textsubscript{4}-NaH\textsubscript{2}PO\textsubscript{4} pH 7.5, 50 mM NaCl, 10% D\textsubscript{2}O) and placed into 5 mm NMR sample tubes. The NMR buffer included detergent when required (1 mM DDM, 1 mM LMPG). NMR spectra malE-en2NTS\textsubscript{1}
were acquired on approximately 20μM malE-en2NTS, ± 100μM NTs, or 30μM en2NTS ± 50μM NTs, with a Bruker Avance 800 MHz spectrometer equipped with a cryogenic probe. 2D 1H–2C SOFAST-HMQC spectra were typically recorded at 298K with spectral widths of 9,615 Hz (1,024 data points) and 8,000 Hz (128 data points) for the 1H and 2C dimensions respectively with a relaxation delay of 400 ms. The 13C carrier frequency was positioned at 17 ppm, and the 1H at 4.7 ppm, while band selective 1H pulses were centred at 2 ppm. Prior to Fourier transformation, data were multiplied by cosine-bells and zero-filled once in each dimension. All NMR data was processed in NMRPipe and plotted in Sparky (Goddard, T.D. and Kneller, D.G., University of California, San Francisco).

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Author Contributions
P.J.S., P.R.G., D.J.S. and R.A.D.B. conceived of and designed the study. P.J.S. conducted all cell free protein synthesis experiments. P.J.S. and F.B. acquired CD data and P.J.S. conducted ligand-binding studies; F.B. expressed and purified receptor from E. coli. P.J.S., F.B., P.R.G. acquired and processed NMR data. P.J.S. drafted the manuscript. All authors reviewed and edited the entire report and approved the final version for publication.

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