Neurotensin receptor type 1: *Escherichia coli* expression, purification, characterization and biophysical study

P.J. Harding*, H. Attrill*, S. Ross*, J.R. Koeppe*, A.N. Kapanidis† and A. Watts*[1]

*Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K., and †Department of Physics, Clarendon Laboratory, Parks Road, Oxford OX1 3PU, U.K.

Abstract

NT (neurotensin) is an endogenous tridecapeptide neurotransmitter found in the central nervous system and gastrointestinal tract. One receptor for NT, NTS1, belongs to the GPCR (G-protein-coupled receptor) superfamily, has seven putative transmembrane domains, and is being studied by a range of single-molecule, functional and structural approaches. To enable biophysical characterization, sufficient quantities of the receptor need to be expressed and purified in an active form. To this end, rat NTS1 has been expressed in *Escherichia coli* in an active ligand-binding form at the cell membrane and purified in sufficient amounts for structural biology studies either with or without fluorescent protein [YFP (yellow fluorescent protein) and CFP (cyan fluorescent protein)] fusions. Ligand binding has been demonstrated in a novel SPR (surface plasmon resonance) approach, as well as by conventional radioligand binding measurements. These improvements in production of NTS1 now open up the possibility of direct structural studies, such as solid-state NMR to interrogate the NT-binding site, EM (electron microscopy), and X-ray crystallography and NMR.

Introduction

GPCRs (G-protein coupled receptors) are a family of integral membrane proteins with seven transmembrane helices that are involved in a range of physiological processes, including cell–cell communication, sensory transduction, neuronal transmission and hormonal signalling [1]. Given that the majority of GPCR-targeted drugs in clinical practice (which represent more than 30% of all drugs) exert their actions on only 30 of the 750 identified human GPCRs, there is significant scope for further drug discovery in this field [1,2]. Paradoxically, a structure of a ligand-binding GPCR is still awaited, and significant technical challenges hamper the routine expression and purification of this class of receptor in an active ligand-binding form.

NT (neurotensin) is an endogenous tridecapeptide neurotransmitter (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) found in the central nervous system and gastrointestinal tract. It is responsible for the activation of the NTS (neurotensin receptor) family [3]. One such receptor, NTS1 (neurotensin receptor type 1), is a member of the GPCR superfamily [4]. Major effects of NT include analgesia, inhibition of food intake, modulation of dopaminergic systems and pituitary hormone secretion, and regulation of digestive function. It also acts as a trophic factor on human colon, pancreatic, prostate and lung cancer cell lines [5]. NTS1 therefore represents a possible target for the treatment of pain, obesity, schizophrenia and cancer. Here, the current status of our efforts to characterize NTS1 in preparation for structural studies is reported.

Optimized expression of NTS1

Rat NTS1 has been expressed previously as a protein fusion with MBP (maltose-binding protein) and TrxA (thioredoxin)–His$_{10}$ in the *Escherichia coli* DH5α strain [6–8]. Expression trials of the NTS1 construct were carried out in *E. coli* DH5α and BL21(DE3) cells, as well as C41(DE3) and C43(DE3)pREP4, two mutant strains derived from BL21(DE3) which have proved useful for the overexpression of several membrane and toxic proteins [9]. Cell pellets were detergent-solubilized, purified by IMAC (immobilized metal-ion-affinity chromatography) and assayed using a radioligand binding assay. The BL21(DE3) and C41(DE3) strains, induced with an IPTG (isopropyl β-D-thiogalactoside) concentration of 0.25 mM, produced the highest level of functional protein per litre of culture, yielding an approx. 3-fold improvement over DH5α. Increasing the IPTG concentration above 0.25 mM resulted in a decreased receptor yield in all cell types, suggesting that expression of the construct at higher rates has a toxicity effect (H. Atrill, P.J. Harding, E. Smith and A. Watts, unpublished work).
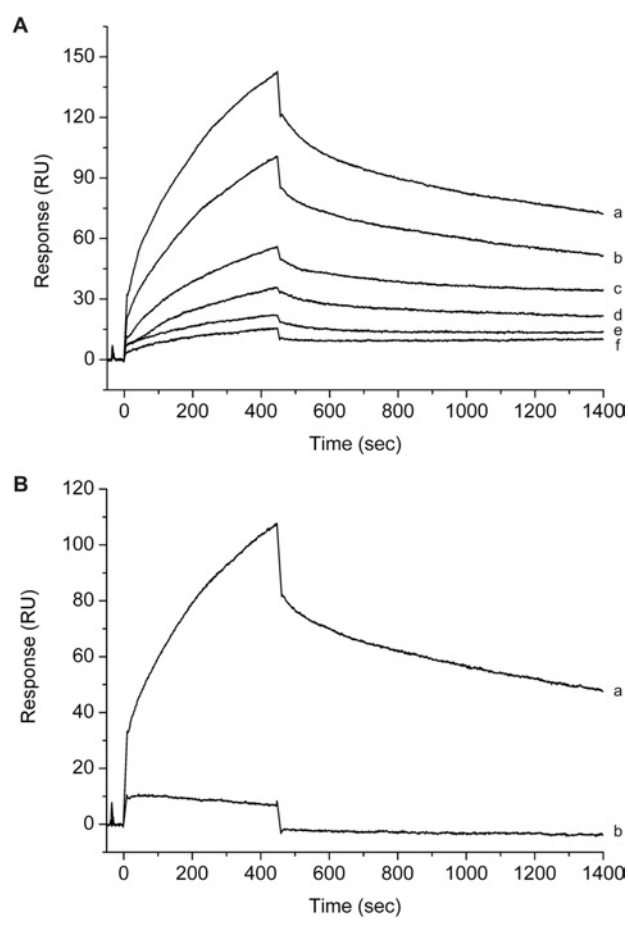
Figure 1 | SPR response curves for the NT/NTS1A interaction using immobilized NT

(**A**) The interaction between detergent-solubilized NTS1A and biotinylated NT. Various concentrations of NTS1A [5.8 nM (a), 2.9 nM (b), 1.5 nM (c), 0.73 nM (d), 0.36 nM (e), 0.18 nM (f)] were injected at 20 μl/min over 250 RU (response units) of immobilized NT and control ligands. The control (scrambled peptide) response is subtracted and therefore only specific binding of NTS1A to immobilized NT is shown. (**B**) Binding of detergent-solubilized NTS1A to the biotinylated NT (a) and control peptide (b) flow cells at a concentration of 2.9 nM. The response obtained for a blank Sensor Chip SA flow cell has been subtracted from both curves, revealing negligible non-specific binding to the control peptide surface.


Figure 2 | Schematic diagram depicting the untagged NTS1B construct [8] and the fluorescence-tagged NTS1C and NTS1Y constructs

NTS1B consists of MBP from *E. coli*, an N-terminally truncated form of NTS1 (T43NTS1), thioredoxin A from *E. coli* (TrxA) and a His$_{10}$ tag. TeV protease cleavage sites are present between the MBP and T43NTS1 moieties and the T43NTS1 and TrxA moieties to facilitate proteolytic removal of the fusion partners. The genes for CFP and YFP were introduced into the NTS1B construct by overlap-extension PCR to yield the NTS1C and NTS1Y constructs. The C-terminal TeV site was moved to a position C-terminal of the fluorescent protein gene.
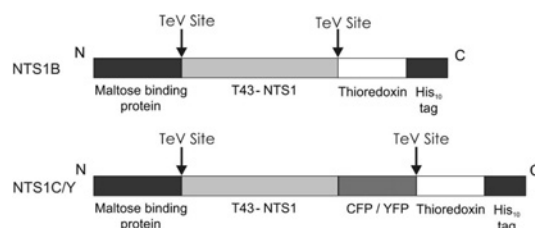
flow cell (Figure 1B), indicating a highly specific interaction of the solubilized receptor with the six C-terminal residues of the NT peptide. An apparent K_d of 1–2 nM was measured, which is similar to the values measured independently using a conventional radioligand ([3H]NT) binding. Such use of the receptor as an analyte is the first example of this approach for a purified detergent-solubilized GPCR, showing potential for high-throughput ligand screening for this class of receptor.
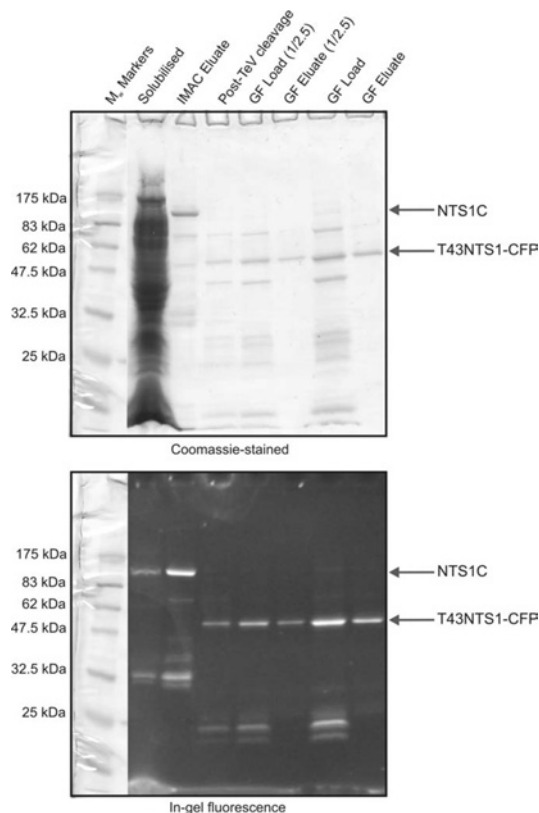
Fluorescent labelling of NTS1 with CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein)

The receptor has also been successfully labelled with CFP and YFP. The genes encoding CFP and YFP were introduced into the NTS1B (MBP–TeV–rT43NTS1–TeV–TrxA–His$_{10}$, where TeV is tobacco etch virus) construct between the T43NTS1 (N-terminally truncated form of NTS1) and TrxA moieties (Figure 2). Proteolytic cleavage of the resulting NTS1C (MBP–TeV–rT43NTS1–CFP–TeV–TrxA–His$_{10}$) and NTS1Y (MBP–TeV–rT43NTS1–YFP–TeV–TrxA–His$_{10}$) constructs after expression and purification yielded NTS1–CFP and NTS1–YFP respectively for use *in vitro* ensemble and single-molecule fluorescence studies to probe the multimerization state of the receptor (P.J. Harding, H. Attrill, S. Ross, E. Smith, G.H. Wadham, J.P. Armitage and A. Watts, unpublished work).

The expression and purification of the receptor was followed using in-gel fluorescence [11] (Figure 3) and radioligand binding. The results indicated that the receptor is both fluorescent and able to bind ligand with high affinity throughout the purification. Given that GFP (green fluorescent protein) and its variants are not fluorescent when expressed on the periplasmic face of the membrane, or in inclusion bodies [12], fluorescence confirms that the protein is being

Ligand binding demonstrated by radioligand binding, and SPR (surface plasmon resonance)

We measured the direct binding of detergent-solubilized NTS1 to immobilized NT ligand using SPR [10]. The redundancy of the seven N-terminal residues of NT in activating NTS1 was exploited by immobilizing N-terminally biotinylated NT to a streptavidin-coated surface. A rigorous specificity control, comprising a biotinylated scrambled peptide, was subtracted from the observed response to yield specific response curves (Figure 1A). Responses observed for the control surface were not significantly different from a blank

Figure 3 | Gels showing how overexpression and purification of NTS1Y was monitored using in-gel fluorescence

Indicated fractions from an NTS1C purification from an *E. coli* C41(DE3) culture were separated on a SDS/12% PAGE gel. In-gel fluorescence was monitored, and the gel was subsequently stained with Coomassie Blue. Various amounts of CFP–His$_6$ were loaded on to the same gel (not shown) to enable quantification of the amount of fluorescent receptor present at each stage of purification. Comparison of the in-gel fluorescence image with the Coomassie Blue-stained image highlights the high sensitivity and selectivity of the in-gel fluorescence technique. Molecular masses (M$_W$) are indicated in kDa. GF, gel filtration; IMAC, immobilized metal-ion-affinity chromatography.

and purification procedures for NTS1, as well as other GPCRs.

Conclusions

Proteins of the GPCR family are predicted to comprise a large proportion of future drug targets. Consequently, further knowledge of the receptor structure, ligand binding and signal transduction mechanisms is required. Given that high-resolution structural techniques, such as solid-state NMR [6,13,14] and crystallography, require milligram amounts of purified receptor in an active ligand-binding form, and the challenges associated with the (over)expression of GPCRs, any optimization of expression levels is clearly advantageous. Here we demonstrate optimization of GPCR expression and purification of NTS1, and demonstrate ligand-binding activity using both conventional radioligand binding assays and a novel application of SPR which enabled the NT–NTS1 interaction to be measured in real time. This approach has potential for application to other suitable GPCR systems, providing insights not only into the kinetics and mechanisms involved in ligand binding, but also into the search for novel ligands using high-throughput screening. Many GPCRs are thought to exist as dimers or oligomers [15]. The successful production of active CFP- and YFP-tagged NTS1 has provided us with valuable tools for the analysis of oligomerization by ensemble and single-molecule FRET (fluorescence resonance energy transfer)-based study of *in vitro* systems.

correctly targeted to the plasma membrane and is folding with the C-terminus of the receptor on the cytoplasmic side. The T43NTS1–CFP product was effectively purified to near homogeneity (Figure 3) and a good correlation between receptor fluorescence and ligand-binding activity was observed during the purification. Further radioligand binding analysis confirmed the high affinity of purified T43NTS1–CFP for NT ($K_d = 0.9 \pm 0.1$ nM). This affinity compares well with affinities for the NT–NTS1 interaction reported previously [7], indicating that the fusion with fluorescent protein does not alter the affinity of the receptor for NT. In addition, comparison of in-gel fluorescence (i.e. total receptor) with activity assay data provides a useful indication of the proportion of receptor which is able to bind ligand and will therefore allow further optimization of expression

References

1 Hill, S.J. (2006) Br. J. Pharmacol. **147**, S27–S37
2 Watts, A. (2005) Nat. Rev. Drug Discov. **4**, 555–568
3 Carraway, R.E. and Leeman, S.E. (1973) J. Biol. Chem. **248**, 6854–6861
4 Tanaka, K., Masu, M. and Nakanishi, S. (1990) Neuron **4**, 847–854
5 Vincent, J.-P., Mazella, J. and Kitabgi, P. (1999) Trends Pharmacol. Sci. **20**, 302–309
6 Williamson, P.T.F., Bains, S., Chung, C., Cooke, R. and Watts, A. (2002) FEBS Lett. **518**, 111–115
7 Tucker, J. and Grisshammer, R. (1996) Biochem. J. **317**, 891–899

8 White, J.F., Loc, T.B., Shiloach, J. and Grisshammer, R. (2004) FEBS Lett, **564**, 289–293

9 Miroux, B. and Walker, J. (1996) J. Mol. Biol. **260**, 289–298

10 Harding, P.J., Hadingham, T.C., McDonnell, J.M. and Watts, A. (2006) Eur. Biophys. J. **35**, 709–712

11 Drew, D., Lerch, M., Kunji, E., Slotboom, D. and de Gier, J.W. (2006) Nat. Methods **3**, 303–313

12 Waldo, G.S., Standish, B.M., Berendzen, J. and Terwilliger, T.C. (1999) Nat. Biotechnol. **17**, 691–695

13 Williamson, P.T.F., Bains, S., Chung, C., Cooke, R., Meier, B. and Watts, A. (2001) in Focus on Structural Biology: Vol. 1. Perspectives on Solid State NMR in Biology (Kühne S.R. and de Groot H.J.M., eds), pp. 191–202, Kluwer Academic Publishers, Dordrecht

14 Watts, A. (2005) Nat. Rev. Drug Discov. **4**, 555–568

15 Milligan, G. (2007) Biochim. Biophys. Acta **1768**, 825–835