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Review

Solution- and solid-state NMR studies of GPCRs and their ligands

Satita Tapaneeyakorn [a], Alan D. Goddard [a], Joanne Oates [a], Christine L. Willis [b], Anthony Watts [a,*]

[a] Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK
[b] School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, UK

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ABSTRACT

G protein-coupled receptors (GPCRs) represent one of the major targets of new drugs on the market given their roles as key membrane receptors in many cellular signalling pathways. Structure-based drug design has potential to be the most reliable method for novel drug discovery. Unfortunately, GPCR-ligand crystallisation for X-ray diffraction studies is very difficult to achieve. However, solution- and solid-state NMR approaches have been developed and have provided new insights, particularly focussing on the study of protein-ligand interactions which are vital for drug discovery. This review provides an introduction for new investigators of GPCRs/ligand interactions using NMR spectroscopy. The guidelines for choosing a system for efficient isotope labelling of GPCRs and their ligands for NMR studies will be presented, along with an overview of the different sample environments suitable for generation of high resolution structural information from NMR spectra.

Contents

1. Introduction

G protein-coupled receptors (GPCRs), the largest group of membrane receptors (~800 in humans), are encoded for by 1% of the open reading frames (ORFs) of the genomes of higher eukaryotes. GPCRs are seven transmembrane helix proteins, connected by three

Abbreviations: β_2AR, β_2 adrenergic receptor; CP, cross polarisation; CXCR1, chemokine receptor; DAGK, diacylglycerol kinase; GPCR, G protein-coupled receptor; MAS, magic angle spinning; MBP, maltose binding protein; NOE, nuclear overhauser effect; NTS1, neurotensin receptor 1; OmpG, outer membrane protein G; REDOR, rotational echo double resonance; RR, rotational resonance; solNMR, solution-state NMR; SSNMR, solid-state NMR; trNOE, transferred nuclear overhauser effect; SPPS, solid-phase peptide synthesis; τ_c^{-1}, tumbling rate

* Corresponding author. Tel.: +44 1865 275268; fax: +44 1865 275234.
E-mail address: anthony.watts@bioch.ox.ac.uk (A. Watts).

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intracellular and three extracellular loops (Fig. 1). GPCRs are activated by a wide range of stimuli, including hormones, neurotransmitters, ions, odorants, and photons of light [1]. The GPCR superfamily is involved in a variety of biological and pathological processes such as development and proliferation [2], neurological disorders [3], angiogenesis [4], metabolic disorders [5], and immune system and inflammation [6]. Consequently, they are one of the largest classes of drug targets to which agonists and antagonists are currently focused. Although approximately 50% of pharmaceuticals target GPCRs, only 10% of the GPCRs, excluding olfactory receptors are targeted by marketed drugs, indicating the possibility that the remaining 90% of GPCRs are available as targets for the treatment of human disease [7].

Based on protein sequence similarity, GPCRs are commonly divided in three distinct families, A, B and C [8,9], but further divisions can be used, depending on the criteria used. For instance, Fredriksson and colleagues have proposed two more families of GPCRs, frizzled/smoothened-like and adhesion-like families on the basis of phylogenetic criteria [10]. All receptors in each family share ≥20% sequence identity and >40% similarity within the seven-transmembrane domains. Family A (rhodopsin-like receptors) comprises approximately 180 liganded GPCRs, 110 orphan GPCRs and approximately 350 olfactory GPCRs, encompassing more than 80% of total GPCRs. Most clinically used pharmaceuticals which target GPCRs are directed at this family [1,11].

It is generally accepted that GPCRs can exist as dimers or as part of larger oligomeric complexes [12], indeed the dimeric form of some receptors has been recently reported [13,14]. However, many studies support monomeric GPCRs as functional units [15–17] and the area is still highly controversial [18].

Structural studies of GPCR-ligand interactions are still challenging due to the difficulty of functional GPCR production and receptor stability. To date, only five inactivated GPCR structures (bovine and squid rhodopsins, β_1AR, β_2AR, and A_{2a}R) have been resolved at high resolution by X-ray crystallography [19–23]. All resolved structures belong to GPCR class A family. These structures show extensive similarity consisting of seven transmembrane spans, an extra small helix on the intracellular side of the membrane and a conserved disulfide bridge (Fig. 1). The structural divergence arising in the extracellular and intracellular regions are likely to result in their specificity of ligand binding and G-protein coupling, respectively. However, despite their importance, the function of these loops is still in debate and can be difficult to resolve structurally. The rhodopsin receptors are activated by light incident on the covalently bound

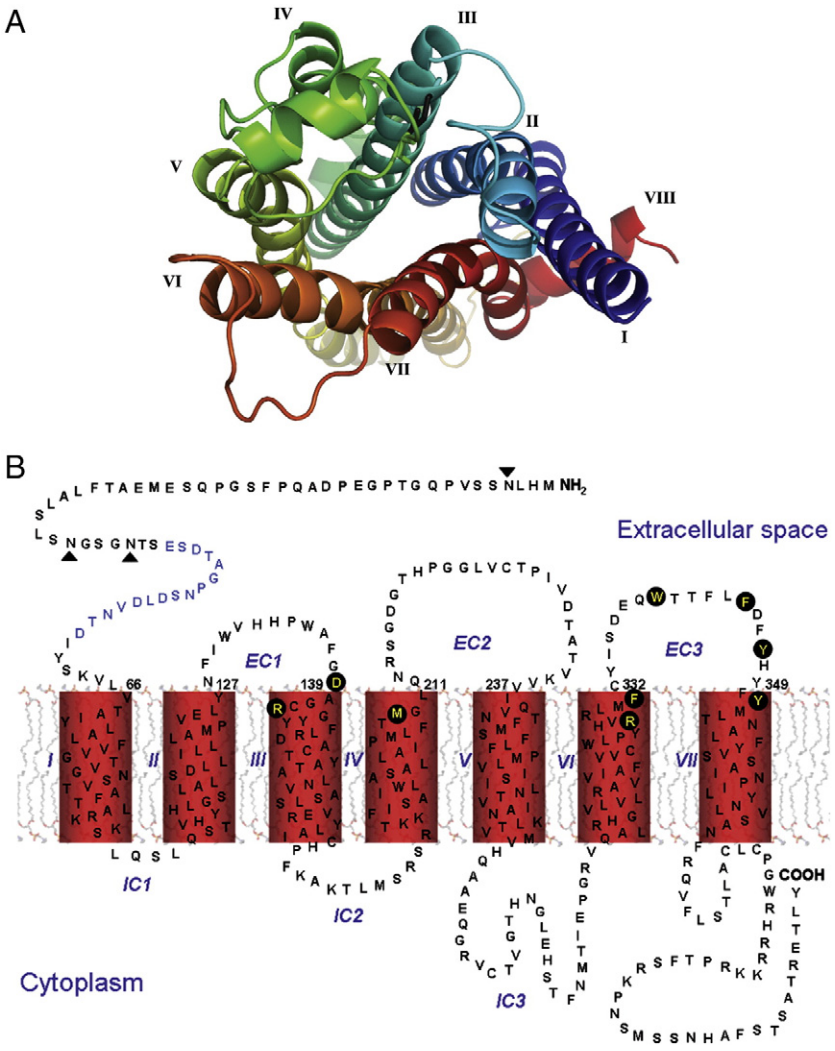

Fig. 1. Homology models based on the limited number of GPCR crystal structures are now being constructed to guide biophysical studies. As an example here, A: a 3D structural model (top view) of NTS1 based on the crystal structures of turkey β_1 adrenergic receptor (PDB ID: 2VT4) and squid rhodopsin (PDB ID: 2Z73): seven transmembrane spans (I–VII), an extra small helix (VIII) in the intracellular side and a conserved disulfide bridge (C142–C225, black sticks) (S. Tapaneeyakorn, unpublished data). B: a 2D structural model of NTS1: residues putatively involved in the binding of neurotensin ligand are indicated by black circles and the N-terminal segment (residues 45–60), important in ligand binding, is shown in blue.

retinal, and the three remaining structures are peptide-activated GPCRs, but none of these latter receptors were crystallised with their native peptide ligands.

Mutagenesis studies have shown that nonpeptide agonists/antagonists interact within the transmembrane segments, whereas peptide ligands bind at the interface between the transmembrane sections and the extracellular loops [24–27]. Therefore, it has been suggested that the binding site of nonpeptide agonists/antagonists to a receptor is different from that of peptide ligands [28]. It is still unclear whether the GPCR-bound conformation of a peptide is structurally related to nonpeptide agonists and antagonists. Peptide ligands bound to receptors belonging to the same family may have different structures, indicating receptor selectivity and specificity of the ligands. The conformation of bound ligand is important for rational drug design for the treatment of diseases caused by GPCR malfunctions. Most GPCR-targeted drugs to date are nonpeptide mimics — they are seldom able to mimic the interactions required to induce a full peptide ligand signal with selectivity, and consequently patients have several side effects from taking these drugs [29,30]. There are many attempts to determine receptor-bound and unbound conformations of peptide ligands in order to understand the differences in receptor binding and activation, with consequences for rational drug design.

Here, the use of NMR spectroscopy for GPCR studies will be presented, including its benefits and limitations. A description of general solution- and solid-state NMR techniques will be also given. In addition, this review will provide guidelines for new researchers who are interested in working on GPCRs, especially with protein-ligand interactions. An overview of expression systems and sample environments to study GPCRs, and their interactions with ligands will be presented, specifically focused towards NMR studies.

2. NMR studies of G protein-coupled receptors

Currently, there are three major approaches, X-ray crystallography, solution-state NMR (solNMR) and solid-state NMR (SSNMR), to study protein-ligand interactions at atomic resolution. Although X-ray crystallography is the method of choice for large molecules, it presents some significant difficulties, for example, producing crystals and obtaining good diffraction, and mostly provides only a static view of inherently dynamic molecules. NMR-based methods have therefore become more important in the study of protein-ligand interactions. These NMR approaches can be applied to weak and transient protein-ligand complexes that are difficult to study by other structural methods [31]. NMR also allows measurement of macromolecular motions at near-atomic resolution, but motions on a variety of time scales and amplitudes, which are essential in providing functional information, are not well understood. In addition, proteins can be studied in a variety of environments, such as different buffer conditions, detergent micelles or bicelles, oriented bilayers, or crystals [32–35]. However, it should be noted that solNMR and SSNMR techniques have been used to date to address specific questions, rather than to resolve three-dimensional GPCR structures [36–38], even though this potential exists. For example, the activation of the β_2 adrenergic receptor (β_2AR) by formoterol induces solNMR spectral changes of [^{13}C]methyl-β_2AR, allowing some limited titration which is interpreted as conformational changes in helices 6 and 7 (Fig. 2) [36].

NMR linewidths, an important factor for NMR data analysis, are normally proportional to the tumbling rate (τ_c^{-1}), so large molecules which have slow tumbling rates ($\tau_c^{-1} \sim 0.75\ kT/\pi r^3\eta$, where r = molecular radius and η = viscosity) will give large linewidths, which preclude structural analysis. In solNMR, the molecule needs to tumble rapidly and isotropically in solution on the NMR time scale (~10^{-1}–10^{-6} s). Thus, all orientational information is lost as a consequence of isotropic tumbling, but fast tumbling averages dipolar interactions to zero and chemical shifts to isotropic values, resulting in sharp NMR resonances.

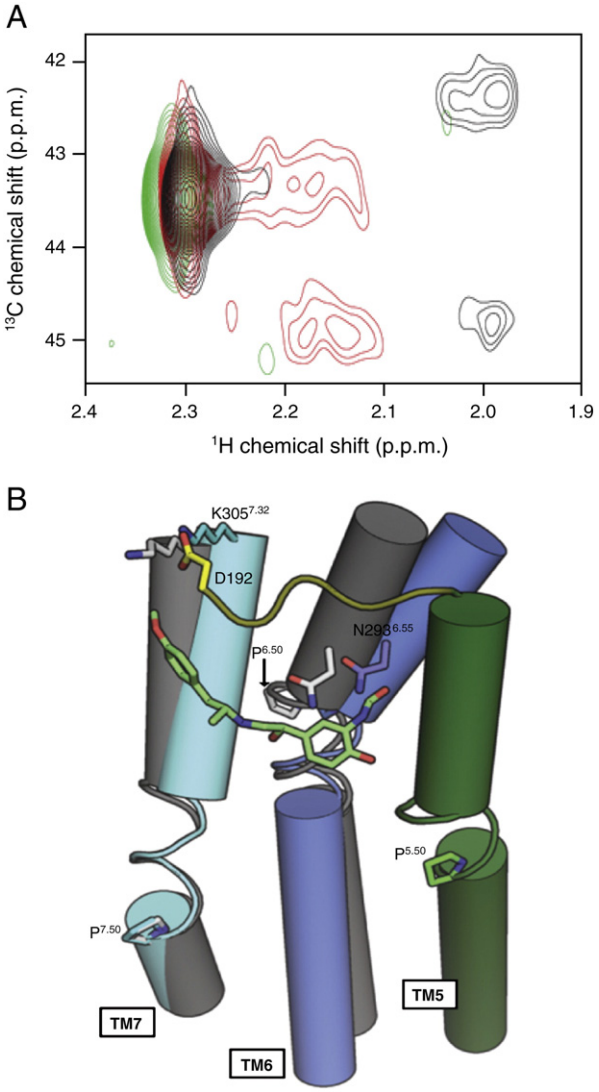

Fig. 2. Structural changes of β_2AR by formoterol. A: the overlay STD-filtered HMQC spectra of unliganded β_2AR (black), agonist formoterol-bound β_2AR (green) and inverse agonist carazolol-bound β_2AR (red). B: a structural model of β_2AR activation by formoterol. Coloured helices, loops and side chains represent the carazolol-bound crystal structure. Grey helices and white side chains indicate the active-state model. Green sticks indicate formoterol and yellow indicates the extracellular loop 2. Adapted from [36] with permission from Copyright owners.

Although transverse relaxation optimised spectroscopy (TROSY) and deuteration techniques provide the possibility to study biomolecules with sizes exceeding a molecular mass of 100 kDa by solNMR [39,40], traditional solNMR is of limited use for structural studies on large proteins, membrane proteins and solid materials, because long correlation times or restricted motions result in incomplete averaging of the anisotropic interactions leading to the broadened spectrum. Also, in the case of membrane proteins, it is generally difficult to keep these proteins active at high concentration at room temperature for the experimental time required to gain spectra with high signal-to-noise ratio and resolution. SSNMR can overcome these averaging limitations to provide structural and dynamic information on proteins and their bound ligands [41,42]. The technique is designed to elucidate structural parameters of slowly tumbling or solid-phase samples at atomic resolution. It is also useful for orientational studies, and has no theoretical limit of size [41,42].

Unlike solNMR, the resolution and sensitivity of SSNMR are affected by the size and orientation-dependence of the nuclear spin

interactions, *i.e.* the chemical shielding and the homonuclear and heteronuclear dipolar spin–spin couplings. These interactions are not averaged to zero, usually generating line-broadening for resonance SSNMR spectra of a static sample. Sample orientation or high spinning of a sample at the magic angle spinning (MAS), together with isotopic labelling, can improve both resolution and signal-to-noise ratio.

SSNMR has been developing rapidly for in particular, the study of membrane proteins and other non-soluble or large biological molecules, since crystals and rapid isotropic tumbling are not necessary. It has the potential to gain accurate internuclear distances and orientations which give information about molecular structures [43]. SSNMR has been employed to provide structural and mechanistic insights of many membrane proteins including GPCRs, reviewed [44–46], for example rhodopsin [37,47], H1 receptor [38], sensory rhodopsin II [48], bacteriorhodopsin [49–52], and phage-coat proteins [53–55].

3. General solution- and solid-state NMR techniques

Nuclear overhauser effect (NOE) related experiments for solution samples provide structural information of protein and a high-affinity protein-ligand complex, orientations of the binding domain and dynamic information of protein-ligand interactions. Assignments of each resonance in the ^{1}H NMR spectrum are required for NOE-related experiments. Since the strength of the NOE is proportional to the internuclear distance (r^{-6}) between two nuclear spins, each cross-peak in the NOE experiment can be converted in to a maximum distance between the nuclei, usually closer than ~5 Å. However, a distance range, rather than a precise value is usually used because the intensity-distance relationship may not be precise due to local dynamics.

In contrast to the NOE, transferred nuclear overhauser effect (trNOE) is the method of choice for the study of low-affinity binding of ligands to protein. However, the conformation of protein-bound ligands can only be determined if the dissociation constant (off-rate) of the ligand from the protein is faster than the longitudinal relaxation rate of the magnetisation of the peptide. The easy execution of trNOE experiments has made it a very popular approach in the past decade [56–58]. Structural information from trNOE experiments can be extended by the measurement of residual dipolar couplings in terms of binding geometries [59].

There are several NMR-based methods to determine the location of ligand binding sites and to extract structural information of a ligand in complex with a protein. Protein-ligand interactions induce changes in a variety of NMR parameters, such as chemical shifts, relaxation parameters, dynamic parameters, diffusion coefficients, saturation transfer differences, and transfer NOEs. Therefore, these parameters can be used as indicators of the interactions (for detailed reviews see [60–62]). For instance, strong association of the ligand with the protein usually leads to chemical shift changes of both protein and ligand (see example in Fig. 3) [63–65]. Also, as linewidth is directly related to apparent molecular weight, if a small ligand binds a protein, its linewidth will broaden [66,67]. These two parameters are most commonly used for probing protein-ligand interactions, providing information about residues involved in interactions, and as a tool for ligand screening in drug discovery [68,69].

The observed interactions from chemical shift perturbations can be confirmed by more precise methods, such as distance measurements. Precise structures at the binding site can be obtained from intra or internuclear distances. As mentioned earlier, MAS has been developed to overcome the problems of analysis of broad spectra of non-isotropically, quickly tumbling samples [70,71]. The basic principle is to spin the sample rotor at 54.7° (the magic angle) with respect to the static field B_0. There are two commonly applied techniques which use MAS combined with recoupling methods in order to get distance information. The first is rotational resonance (RR), which is used to measure the homonuclear dipolar coupling between spin ½ pairs such as ^{13}C–^{13}C and ^{1}H–^{1}H pairs [72,73]. The second technique, rotational

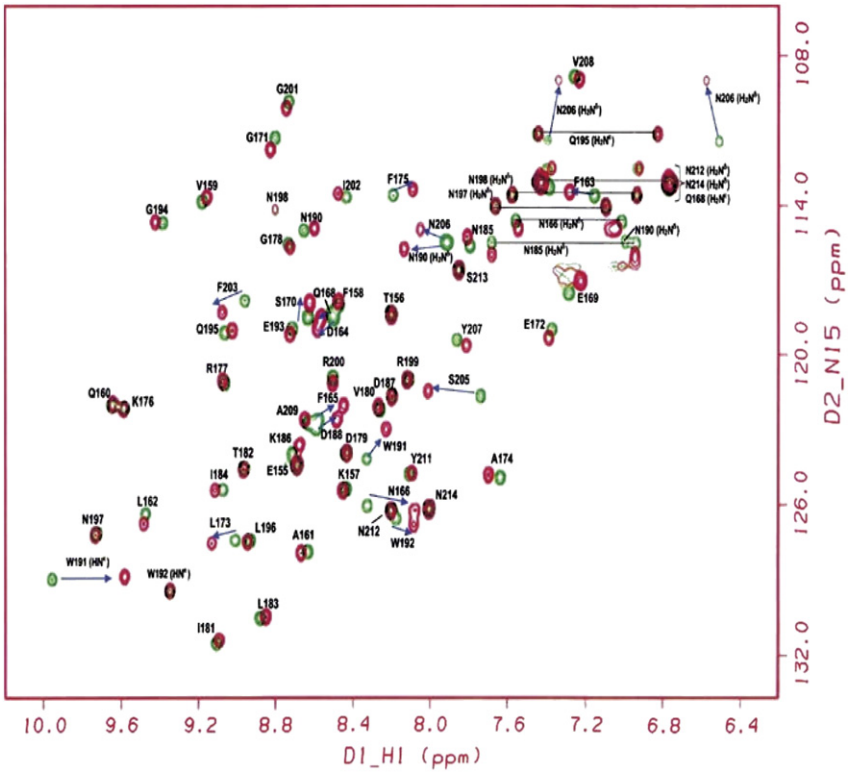

Fig. 3. A superposition of ^{1}H/^{15}N-HSQC spectra for Sem-5 C-SH3 in the absence (green contours) and presence (red contours) of peptide. Significant chemical shift changes upon ligand binding are indicated by blue arrows (adapted from [65] with permission from Copyright owners).

echo double resonance (REDOR) is used to measure the heteronuclear dipolar coupling between spin ½ pairs such as ^{13}C–^{15}N and ^{13}C–^{19}F pairs [74].

These techniques have been employed for structural determination of several proteins. For instance, ^{13}C–^{13}C RR and ^{13}C–^{19}F REDOR techniques were employed to investigate the molecular mechanism of transmembrane signalling of the serine bacterial chemoreceptor upon ligand binding [75,76]. The RR experiments were also used to measure the distance between the positively charged nitrogen and the carbonyl once the uniformly labelled acetylcholine bound to the nicotinic acetylcholine receptor. A distance of 5.1 Å was measured indicating a bent conformation of bound acetylcholine [77]. If the spectrum is dominated by natural abundance background from buffer, lipids or large receptor molecules, the problem can be resolved by introducing double quantum filtering techniques (Fig. 4), for example C7 [78], POST-C7 [79], INADEQUATE [80], and $R14_2^6$ [81]. The one drawback for site-specific spin labelling for distance measurements is the low signal intensity compared to natural abundance of the same spin type in the system. However, the potential use of a nitroxide spin label has been recently explored to enhance the NMR signal through dynamic nuclear polarisation (DNP) [82–84].

4. Isotope labelling

Assignments and sensitivity enhancement can be achieved through chemically directed isotropic labelling. Additionally, label selection will be determined by the questions being addressed about structure and dynamics of proteins, for both solNMR and SSNMR spectroscopy. Assignment of resonances can be made for homonuclear and/or heteronuclear spectra depending on labelling strategies and available NMR samples, for example, homonuclear 2D spectra for unlabelled proteins, ^{15}N heteronuclear spectra for ^{15}N labelled proteins, or triple resonance spectra for $^{15}N/^{13}C$ doubly labelled proteins.

NMR signals from protons, as well as ^{13}C and ^{15}N (spin = ½) are commonly detected in biological NMR assignments from solNMR experiments. However, in proton detection considerable difficulties are encountered in SSNMR since strong dipolar couplings (~100 kHz) between protons give rise to structureless broad lines, even when using sophisticated resolution enhancement techniques combined with fast (>50 kHz) MAS of the high magnetic field. Since ^{13}C and ^{15}N nuclei have smaller dipolar couplings (~10 kHz) and larger chemical shift range (~200 ppm) than ^{1}H nucleus, they give rise to much better resolved spectra. Therefore, they are the nuclei of choice for detection in SSNMR experiments [85]. In these cases, cross polarisation (CP) from ^{1}H to less sensitive nuclei helps to enhance sensitivity [86].

Quadrupolar nuclei (spin>½), such as ^{19}F, ^{31}P and ^{2}H for example, are less-common nuclei for NMR studies of biological molecules. However, the sensitivity of ^{19}F and ^{31}P is very high as they have 100% natural abundance leading to a lack of background signals. Natural isotope labelling with ^{31}P is useful for the studies of GPCR-lipid or ligand-lipid associations [87–89]. Deuterium, ^{2}H, has such a low natural abundance and a short longitudinal relaxation time so deuterium detection is not routine in traditional NMR experiments. However, deuterium labelling does increase the resolution and sensitivity in multidimensional NMR experiments and deuterated solvents are often used to avoid solvent-signal interference in the proton spectrum (reviewed in [90]). In SSNMR, it is still unclear whether deuteration has any effect on spectral linewidths of proteins as a limited number of studies have revealed inconsistent results [49,91,92] and none have been reported for GPCRs.

A peptide ligand and protein can be isotopically labelled in two different ways, either uniform or selective/specific labellings, depending on the purpose of the experiment. Normally, selective isotope labelling is widely used to aid assignment and structure determination of large proteins because it reduces unwanted peaks and decreases the spectral crowding. In addition, site-specific labelling of spin pairs can be selectively detected for addressing a specific question of interest.

There are two major methods for producing isotopically labelled materials, solid-phase peptide synthesis (SPPS) and expression (see

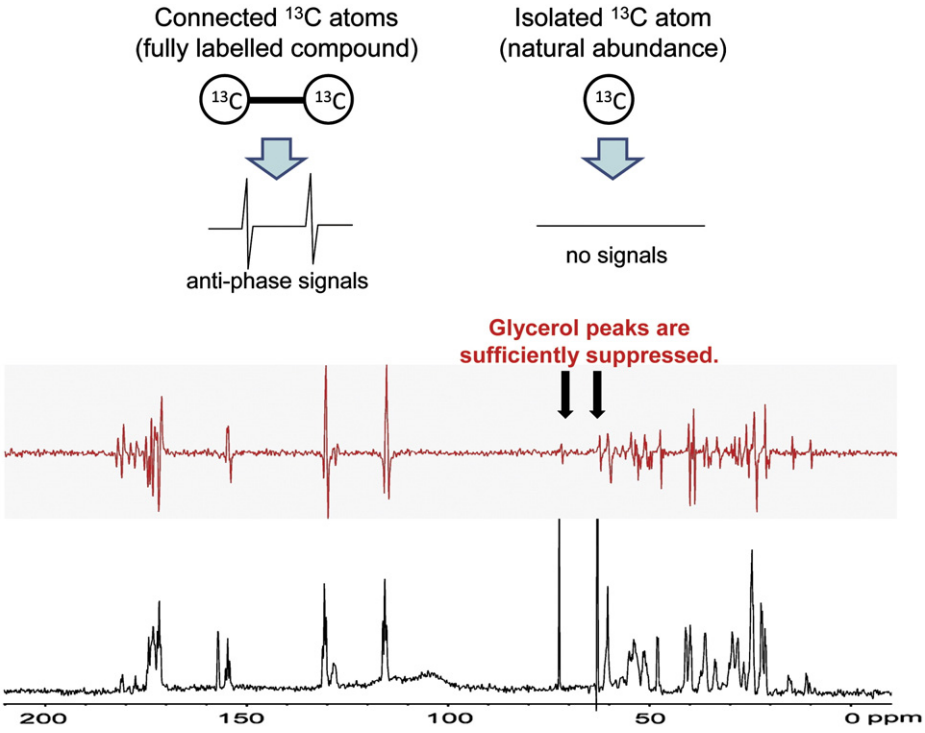

Fig. 4. 1D ^{13}C spectra of uniformly ^{13}C, ^{15}N labelled neurotensin ligand in detergent buffer (50 mM Tris–HCl, pH 7.4, 20 mM KCl, 1% DDM/0.01% CHS, 1 mM EDTA, 5% glycerol) with (red trace) and without (black trace) double quantum filter (INADEQUATE). INADEQUATE suppresses signals from natural abundance and results in anti-phase line shapes (S. Tapaneeyakorn, unpublished data).

later discussion). The SPPS method is suitable for short amino acid sequences, so it has been widely used for the synthesis of small peptides and proteins including labelled peptide GPCR-ligands [64,93,94]. The benefit of SPPS is that highly specific local information can be obtained through judicious labelling strategies, where uniform labelling may not yield such specificity due to spectral overlap [72]. However, labelled amino acids are very expensive for the SPPS method which requires necessary protection of reactive groups (for example Fmoc and Pbf protection of α-amino and side-chain amino groups of Arg, respectively) and can be wasteful of labelled materials. Therefore, an expression system is the method of choice when generating labelled samples in high yield in a cost effective manner, using more economic ^{15}N and/or ^{13}C sources, particularly for large biomolecules.

4.1. Comparisons of expression systems

A large amount (0.1–5 mg in ~100–300 μl) of a GPCR is required for NMR studies. With the notable exception of rhodopsin, GPCRs are generally expressed at low levels in their endogenous environment. Therefore, it has been necessary to develop heterologous expression systems to achieve the high yields of protein. Incorporation of isotopic labels into the receptor is an additional requirement for performing target-based NMR experiments, and the process can be easier in some systems than others. A number of reviews in the past few years have focused on expression systems for GPCRs in detail [95,96]. Here, we aim to give an overview of the different systems and to focus on their application to produce samples suitable for NMR analysis. The same systems can obviously also be used to produce labelled peptide ligands.

4.2. Escherichia coli

E. coli is an extremely well-established system for heterologous protein production. Despite numerous advantages (Table 1), there has been a very low success rate of expressing GPCRs in *E. coli*. The most likely explanations for the problems encountered result from the fact that *E. coli* is a prokaryote whereas all identified ligand binding GPCRs are eukaryotic. Some of the key limitations are outlined in Table 1. Although these problems may appear daunting, there have been a number of successes in producing functional GPCRs in *E. coli* [97–103]. Many of these studies have adopted the use of fusion proteins to increase the solubility of the overall protein and to help to orientate it in the *E. coli* membrane. For example, receptors can be fused to the C-terminus of *E. coli* maltose binding protein (MBP) in which the signal peptide of MBP acts to direct the EC1 loop of the GPCR to the periplasm [104–106]. Additionally, fusion of a soluble protein such as thioredoxin to the C-terminus of the GPCR can assist in expression [105,106]. Such fusion proteins can be cleaved from the expressed and purified GPCR by the inclusion of judicious protease sites. However, expression of functional GPCRs in *E. coli* typically involves a "trial and error" procedure in which different fusions should be investigated and induction conditions varied to balance active protein production with culture volume and viability.

Despite these successes, the expression levels of GPCRs in *E. coli* are still relatively low compared to those desirable for biophysical studies, although this is partly mitigated by the scalability of *E. coli*. It should be noted, however, that there is not necessarily a linear relationship between culture volume and yield of active protein. One of the highest yields reported is that of neurotensin receptor 1 (NTS1) (~800 μg of active receptor per litre of culture), but this is still below the desired mg per litre levels [105].

Once a GPCR has been expressed in *E. coli*, this does provide the advantage that isotopic labelling is extremely well developed. For example, minimal media recipes allow uniform labelling with ^{15}N using ammonium salts, ^{13}C using glucose or glycerol and ^{2}H using deuterated water. It should be noted that in the majority of cases, protein yield per litre of media and/or gram of wet cell pellet drops significantly (can be <20%) when *E. coli* strains are grown in labelled minimal media. It is therefore desirable to maximise expression levels both for factors of scale and cost.

A variety of approaches have been adopted to increase the yield in minimal labelled media. These include addition of fresh labelled nutrients upon induction [107] or growth in unlabelled media prior to induction followed by harvesting and resuspension in labelled media upon induction [108]. It should be noted that deuteration can be difficult to achieve due to the inability of some strains to grow on D_2O. Therefore, a process of adaptation is often conducted in which cells are grown on increasing concentrations of D_2O up to >90%. Once cells have been adapted they can be stored as glycerol stocks [109]. This adaptation may not, however, be required as certain strains are capable of immediate growth on >90% D_2O.

Additionally, it is possible to label specific amino acids by including their isotopic variants in minimal media recipes. It is important to consider the biosynthetic pathways of *E. coli* as some steps are bidirectional (e.g. C2 metabolism) and may result in unwanted incorporation of labels [109]. It is also possible to use *E. coli* mutant strains which lack the biosynthesis genes for the amino acid of interest, hence ensuring incorporation of the labelled residues. Processes used for incorporation of labelled amino acids can also be applied for the incorporation of non-natural amino acids such as fluorinated derivatives. More complex labelling strategies include site-specific backbone deuteration. This can be achieved by synthesis of a particular $^{15}NH–C\alpha–^{2}H$ amino acid which is then incorporated into recombinant proteins using an appropriate auxotrophic *E. coli* strain [109,110]. It is also possible to deuterate specific amino acid side chains by incorporation of modified amino acids or by addition of appropriately labelled precursors. Other strategies include labelling of specific amino acids in a perdeuterated background, selective complete amino acid protonation and selective methyl group protonation which are comprehensively reviewed elsewhere [109,111].

4.3. Yeast

Yeast provides an attractive host for heterologous expression of GPCRs. The advantages and disadvantages are detailed in Table 1. Three yeast species form the backbone for heterologous protein expression. *Pichia pastoris* can be cultured to a high cell density and possesses expression systems which can be controlled by methanol. A recent study has optimised expression of a number of GPCRs in *P. pastoris* [112]. Expression in *Saccharomyces cerevisiae* can be controlled by galactose induction and many GPCRs have been functionally expressed in engineered *S. cerevisiae* strains [113], although not necessarily at levels which would enable purification of sufficient quantities for biophysical studies. Finally, *Schizosaccharomyces pombe* has expression vectors which can be controlled by thiamine and can express functional heterologous GPCRs [114,115], although there are no reports to date of purification of functional heterologous receptors from this system.

The combination of a number of strategies is often required for isolation of large amounts of active receptor. As an example, *S. cerevisiae* has been optimised as a host for high-level expression of the human A_{2a} adrenoceptor. Although functional coupling of this receptor to the signalling pathway in modified yeast cells has been reported [116], a combination of a variety of developments was necessary to produce sufficient protein levels for biophysical study [117]. The receptor was integrated into the genome in multiple copies and was fused to GFP at the C-terminus and also encoded a decahistidine tag to enable purification. After inclusion of cholesterol during the purification process, active receptor could be purified in mg per litre quantities, one of the highest reported yields from any heterologous system [117].

Table 1
Comparison of GPCR expression systems for NMR.

Expression system	Advantages	Disadvantages
E. coli	Inexpensive	Lack of post-translational modification e.g. glycosylation is required for some GPCR-ligand interactions e.g. [181–183]
	Ease of culture	Lack of some eukaryotic membrane components including cholesterol can affect receptor activity
	Genetic flexibility	High level expression may result in formation of inclusion bodies; limited success in refolding GPCRs [91,184–186]
	Strains optimised for protein expression	Different codon usage (overcome by expression of rare tRNAs)
	Very flexible isotope labelling strategies	Low success rate of active GPCR expression
	High scalability, although there is not necessarily a linear relationship between scale and yield	
Yeast	Inexpensive	Endogenous GPCRs and G proteins which may interfere with expression and purification
	Ease of culture	Different membrane composition
	Genetic flexibility	Relatively thick cell wall may impede purification
	Good scalability	Labelling strategies more limited than some systems
	Cellular compartmentalisation	
	Eukaryotic post-translational modifications	
	Can co-express accessory proteins [187]	
Baculovirus/Insect cells	Eukaryotic; nearly all post-translational modifications are identical to mammalian cells	Glycosylation is different to animal cells
	Most GPCRs expressed are active	Membrane is higher in unsaturated fatty acids and lower in cholesterol [188] which can be key in GPCR activity e.g. [189,190]
	Insect cells are semi-adherent and also grow in suspension allowing good scalability e.g. fermenter growths	Requires high virus titre
	Commercialisation has reduced costs	Cultures are only stable for ~1 month due to accumulation of defective virus particles
		Limited labelling strategies available
Mammalian cells	Native cellular environment	Relatively expensive
	Correct trafficking and folding	Possible complications from endogenous receptors and signalling components
	Correct membrane environment	Difficult to scale
	Correct post-translational modification	Few labelling strategies available
	Most GPCRs expressed are active	Poor scalability
Cell-free expression	Only the protein of interest is produced	Relatively expensive
	Can express toxic proteins	May require optimisation of detergents and membrane mimetics
	Can include detergents and membrane mimetics	Low success rate of GPCR expression to date [191,192]
	Yields >1 mg/ml reaction [191]	
	Complete labelling control	

P. pastoris remains one of the most common hosts for high-level expression of proteins. The ability of the yeast to grow in a defined minimal medium allows uniform labelling with ^{15}N, ^{13}C (e.g. [118]) or ^{2}H [119]. Additionally, amino acid selective labelling is possible [120] with the advantage that prototrophic *P. pastoris* strains can be used, eliminating the drop in protein expression levels normally seen with auxotrophic strains. Although many of the labelling strategies available in *E. coli* can theoretically be applied to *P. pastoris*, development of appropriate techniques is not yet complete.

4.4. Baculovirus/insect cells

Insect cells are used in conjunction with a baculovirus which allows transfection of DNA encoding the GPCR of interest into the cell. Insect cells such as the commonly used Sf9 cells from *Spodoptera frugiperda* have numerous advantages but also some important disadvantages for GPCR expression (Table 1). Insect cells can produce levels of GPCRs comparable to other heterologous expression systems. A comprehensive analysis of expression conditions for sixteen GPCRs was performed [121] and, although good expression of a number of receptors was achieved, there was a large degree of variability between receptors and even for the same receptor in different cell lines. As with most heterologous expression systems there were no hard and fast rules as to conditions which were favourable for expression of active receptor and individual optimisation again appears key for receptor expression in this system.

Labelling of proteins produced in animal cells is inherently more complex than in *E. coli* or yeast. This is largely due to the need for a defined culture medium which can be supplemented with appropriately labelled precursors and amino acids. However recent advances have allowed labelling of certain amino acids [122] and also to uniformly label entire proteins in quantities suitable for NMR [123]. However, there is still significant progress that will have to be made for the baculovirus/insect cell system to offer the wide range of labelling strategies available in other systems.

4.5. Mammalian cells

Mammalian cells provide numerous advantages for GPCR expression (Table 1). However, until recently, heterologous expression of GPCRs in mammalian cells was largely used for pharmacological and functional analysis of the receptors and hence was not optimised to give expression levels suitable for purification of significant amounts of protein.

It is possible to express proteins transiently in mammalian cells (without selection) and also to provide stable expression via a selective process. However, high-level expression of GPCRs in stable cell lines can often be toxic. A common strategy for expression of receptors in mammalian cells is to use Semliki Forest Virus (SFV) vectors. This has numerous benefits including the ability to generate rapidly high-titre stocks, a wide range of host cells and good expression of GPCRs. A recent study used modified SFV vectors to express over 100 different GPCRs in mammalian cells lines including CHO and HEK293 [124]. Approximately 95% of receptors were expressed in an amount detectable by western blotting with 70% expressed at a level suitable for biophysical studies. Additional optimisation of receptor expression was conducted for a number of receptors, with the adenosine A_{2a} receptor being expressed at >250 pmol/mg (equivalent to >2 mg per litre) as determined by ligand binding studies [124]. Although mammalian expression

systems for GPCRs are relatively costly and time-consuming, if they are scalable they have the promise to provide suitable concentrations of active, correctly modified receptors for biophysical studies.

As mentioned previously, labelling of proteins produced in animal cells is inhibited by the requirement for difficult-to-make expensive media. It is possible to uniformly label proteins produced in this manner [125,126]. It is also possible to label only the backbone residues of amino acids with ^{13}C, ^{15}N and ^{2}H [127]. Techniques such as SILAC (Stable Isotope Labelling by Amino Acids in Cell Culture) have been applied to mammalian cells e.g. [128,129]. In this process, essential amino acid/s containing the appropriate isotopic label are included in a cell culture medium which lacks these amino acids and all proteins produced within the cell incorporate these amino acids. This can be applied to nearly all types of cells including primary cells [128].

4.6. Cell-free expression systems

Cell-free expression offers a unique tool in preparation of protein samples for biophysical studies. The near total control over labelling strategies allowed by this system, along with other advantages (Table 1) makes this potentially one of the most significant advances in protein production in recent years (reviewed [130,131]). A key component of cell-free expression systems is the cell lysate used as the basis for the reaction mixture. This is commonly from either an *E. coli* or eukaryotic source. Whilst the *E. coli* lysates generally provide a higher level of protein production, eukaryotic derivatives promote correct folding of eukaryotic proteins, a significant consideration in the production of GPCRs. The advantages and disadvantages are basically identical regardless of the source of lysate and are detailed in Table 1.

As mentioned previously, cell-free expression systems have the highest flexibility of isotopic labelling. As well as incorporation of ^{15}N- or ^{13}C-labelled amino acids, it is also possible to include a single labelled amino acid into the reaction mixture. In this case the amino acid can be labelled either with ^{15}N or ^{13}C. Additionally, it is possible to label one amino acid with ^{15}N and another with ^{13}C to allow assignment of pairs of amino acids. Furthermore, by using engineered tRNA molecules it is possible to attach a single labelled amino acid at a specific position [132]. A strategy of particular interest for membrane proteins is the specific labelling of Ala, Phe, Gly, Ile, Leu and Val which account for ~60% of residues within transmembrane helices [133]. Although most of these techniques have not yet been applied to GPCRs generated by cell-free expression, it is clear that this system has massive potential for NMR analysis of such receptors.

4.7. Choosing the right expression system

It is difficult to argue that one system is vastly superior to any other for expression of GPCRs, each having distinct advantages and disadvantages and all systems require some degree of optimisation. If active receptors can be produced in *E. coli* this allows labelling and scalability. However, eukaryotic systems may be required to produce functional GPCRs, in which case yeast provides a good alternative to *E. coli*. Although it is more difficult and expensive to produce large quantities of labelled protein in insect or mammalian cells, there is a high likelihood that the receptors will be active. Finally, the future looks bright for cell-free expression systems which provide the ultimate control over labelling strategies but are currently limited by scalability and a low number of GPCRs which have been expressed successfully.

4.8. Ligand expression

It is possible to choose an expression system from the previous discussion to produce isotopically labelled peptides, but labelling limitations of the system used must be considered. Most short peptides have been produced in *E. coli*. However, short peptides often express poorly so are normally produced as a fusion with another protein which can then be cleaved after purification.

Some examples of such protein fusion are thioredoxin [134], outer membrane protein A [135], glutathione S-transferase [136] and the GB1 domain of Streptococcal protein G [137–139]. It is often desirable to include a linker region between the fusion protein and the peptide and also an enzymatic or chemical cleavage tag. Using these methods it is possible to produce good yields of peptides e.g. 21.5 mg per litre of GLP1 peptide fused to CMFH domain in rich media [140]. Yields often drop in minimal media although a yield of 48 mg per litre has been reported for CMPcc fused to GB1 [137]. One aspect to consider with the production of fusion proteins is the possible requirement for a free amino or carboxy terminus for full activity of the ligand. For example, neurotensin [141] and angiotensin [142] both require a free-COOH group for maximal activity.

5. Sample environments

Choosing the appropriate environment in which to carry out NMR studies of GPCRs and their ligands is a critical factor in the generation of high resolution data. Obtaining homogeneous sample preparations leads to improved linewidths and therefore spectral resolution, while heterogeneous samples can result in artifacts such as unexpected peaks and peak doubling. Protein samples can be prepared in a variety of states for both solNMR and SSNMR; however, despite numerous studies no global parameters for sample preparation currently exist. Therefore, the most suitable conditions for each protein have to be determined empirically. In terms of GPCRs and their ligands the main parameters involve the physical state of the sample (e.g. microcrystalline/solution), as well as selection of the appropriate solubilising agent (e.g. detergent micelles/lipid bilayers). Both of these factors can strongly influence protein stability and spectral resolution. Below we summarise the different sample environments that can be applied to NMR studies of GPCRs and receptor/ligand interactions along with relevant examples from the literature (see also Fig. 5).

5.1. Detergent micelles and organic solvents

Micellar detergent solutions have been used extensively in NMR experiments on membrane proteins; and are typically the solubilising agent of choice in solNMR. In a micelle the hydrophobic regions of the protein are shielded from the aqueous environment by the surrounding detergent molecules thus preventing protein aggregation. Choosing a suitable detergent can be challenging and generally requires a trial and error approach; some such as DPC and LPPG have gained more popularity than others (for a detailed assessment of detergents for solNMR see [143]). While detergents make good solubilising agents for solNMR they may not mimic closely enough the natural environment of the protein in the bilayer particularly when considering oligomer formation and receptor/ligand binding. Furthermore, detergents increase the effective size of the protein molecule leading to slower tumbling times and subsequent line-broadening. Slow tumbling times restrict the maximum size of the protein for structural studies to ~40 kDa, although assignment of larger proteins has been achieved for example with KcsA [144], Given this size limitation, studies of GPCRs in micelles have essentially been limited to small fragments of the proteins, typically containing one or two transmembrane helices [145–148], although some initial studies have been attempted with the full-length vasopressin V_2 receptor [149,150]. Furthermore, peptide ligands are often studied in a micelle environment independently of the receptor such as Leu-5-enkephalin [151] and dynorphin A [152]. Receptor/ligand interactions however can be difficult to study in micelles on high-affinity systems (such as the interaction between neurotensin and its receptor NTS1 K_d of ~1 nM [13,106,153]) as the interaction timescale involved is too fast for solution measurements.

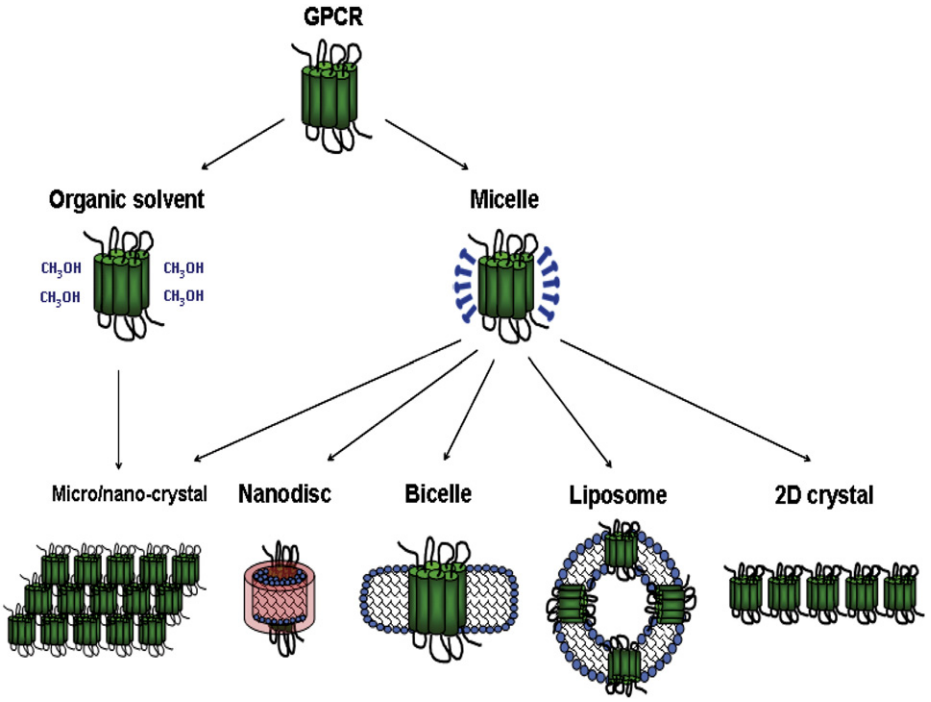

Fig. 5. Schematic representation of different sample environments for NMR studies of GPCRs.

Organic solvent mixtures such as chloroform:methanol or TFE have been utilized as solubilising agents for membrane proteins with a smaller size (compared to a protein micelle complex) leading to more rapid tumbling [13,154–156]. However, in general the biological relevance of an organic solvent environment is questionable as it may induce non-native structural rearrangements in the protein, particularly if there are large extramembranous regions [157,158]. Notable examples of studies involving GPCRs again are restricted to small domains including a 73 residue fragment of the yeast pheromone receptor Ste2p [159], a 40 residue fragment of the human cannabinoid receptor-1 [160] and the C-terminal of the human β_2AR [161].

Although both micelles and organic solvents may not represent a native environment, protein samples can often be prepared in micelles at higher concentration particularly compared with liposomes where random orientation can result in approximately half of the protein unable to bind ligand. Furthermore, solid-state samples can be easily prepared from micellar solutions simply by freezing (to below −50 °C) [93,94]. The conformation of bradykinin bound to human bradykinin B2 receptor in DDM has been recently proposed [94] (see Fig. 6). Detergents and organic solvents can also form a crucial parameter in protein reconstitution into lipid bilayers and precipitation of micro/nanocrystals (see later discussion) for SSNMR experiments.

The development of novel types of solubilising agents in recent years such as short amphipathic polymers known as amphipols, along with reversed micelle systems may provide new possibilities for solNMR investigations of membrane proteins including GPCRs [162–164].

5.2. Freeze-dried or microcrystalline samples

Lyophilised protein samples were originally required for SSNMR experiments; however the freeze-drying process is thought to result in secondary structure alterations leading to rather heterogeneous samples, which do not yield spectra of high enough resolution for structural studies [165]. Samples can be partially rehydrated which, in the case of some soluble proteins such as lysozyme and ubiquitin, leads to higher spectral resolution [166]. As with other sample preparation parameters hydration effects must be tested experimentally. SSNMR samples can additionally be prepared by simply freezing the protein containing solution to below −50 °C as in the case of the neurotensin GPCR ligand. Here, the receptor-bound form of the ligand

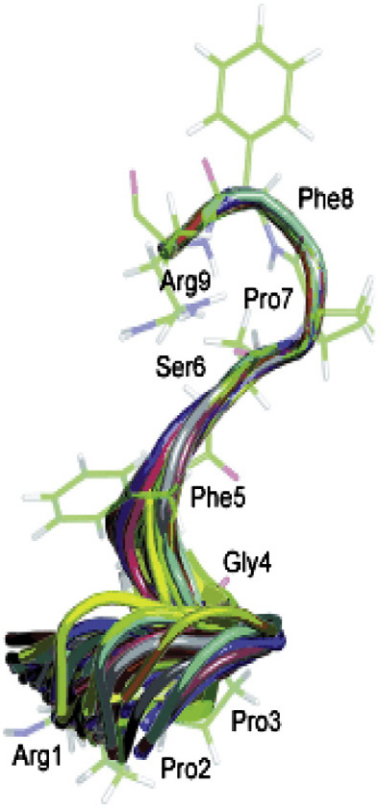

Fig. 6. Backbone model of the neuropeptide bradykinin when bound to the human G-protein-coupled bradykinin B2 receptor in DDM determined by SSNMR spectroscopy at −73 °C. The calculation of the set of 100 superimposed structures was derived from torsion-angle constraints based on ^{13}C chemical shifts (adapted from [94] with permission from Copyright owners).

was determined in the presence of lipid-reconstituted receptor and ligand at −80 °C [93].

Micro/nanocrystalline samples of proteins for SSNMR are prepared by tightly controlled precipitation which often requires extensive optimisation to obtain samples with narrow linewidths [165]. SSNMR techniques however, can make use of much smaller lower quality crystals not accessible to X-ray experiments and have been applied to a number of studies of soluble proteins [167–170], and the membrane protein diacylglycerol kinase (DAGK) [171]. As yet no studies of microcrystalline samples of GPCRs have been reported.

5.3. Lipids

Given that membrane proteins function within a bilayer environment it is more biologically applicable to be able to carry out structural investigations in lipids. SSNMR is ideally suited to such samples, since size limitations with solNMR preclude the use of bilayers or liposomes. Some membrane proteins can be investigated directly in cell membrane preparations such as the photoreceptor bacteriorhodopsin from *H. salinarium*, where the high receptor concentration naturally forms ordered 2D crystals. Most membrane proteins however are not present at such high concentrations *in vivo* so crystal formation is typically induced by lipid reconstitution from micellar solutions; requiring careful optimisation of parameters such as protein lipid ratio as well as lipid/detergent type [172,173]. Such crystals allow the protein to be studied in a native environment preserving structural arrangements which may be critical for regions involved in ligand binding for example. Proteins reconstituted into lipid vesicles are amenable to SSNMR studies although investigations with DAGK and the outer membrane protein G (OmpG) suggest that vesicles give worse resolution spectra than nanocrystalline or 2D samples [171,174] (Fig. 7).

Protein samples can be prepared in liposomes and subsequently deposited on a glass slide, or induced to form bicelles. Both deposited bilayer films and bicelles have the advantage that they can be aligned in a magnetic field which means that orientational constraints can be derived (see an example in Fig. 8); useful in both the study of the membrane protein and also resulting in the alignment of a bound ligand. Indeed, SSNMR experiments performed in a lipid environment are ideally suited to investigations of ligand conformation in the receptor-bound form, a key factor in the development of novel drugs. Investigations of rhodopsin, CC-chemokine receptor 5 and a fragment of the human cannabinoid receptor have been undertaken in bilayers [175–177]; and bound ligand conformation examined with the neurotensin receptor and the human sweet receptor [93,178].

Bicelles were originally developed in the early 90s [179] and are mixtures of long-chain and short-chain phospholipids (or sometimes detergents) which form discrete elongated complexes. The long-chain lipids make up the planar surface with the short-chain lipids or detergents forming the curved ends of the bicelle. The extended planar bilayer region is the main distinguishing feature of a bicelle over a micelle, resulting in considerable differences in curvature stress, thus bicelles are expected to represent a more native-like environment for structural studies [180]. Bicelle size is controlled by the ratio of long and short lipids (for detailed sample preparation see [181]), higher ratios of long chained lipid leads to larger bicelles (500 Å) which can be aligned in a magnetic field, while smaller bicelles (80 Å) result from lower ratios. The ability of large bicelles to orient in a magnetic field can be exploited by SSNMR techniques, whereas smaller bicelles are suitable for solNMR measurements. This means that both solNMR and SSNMR measurements can be made in the same environment allowing for generation of complementary data and method validation [182–184]. GPCRs including the chemokine receptor (CXCR1) have been successfully reconstituted into bicelles using a variety of techniques [185–189].

A novel lipid membrane mimetic termed a nanodisc is rapidly gaining in popularity for NMR applications. Nanodiscs are discoidal lipid bilayers, smaller than bicelles (20–50 nm), and held together by a surrounding scaffold protein of high density lipoprotein (for detailed reviews see [190,191]). These discs provide interesting new potential for structure determination of membrane proteins indeed β_2AR has recently been incorporated into nanodiscs [192].

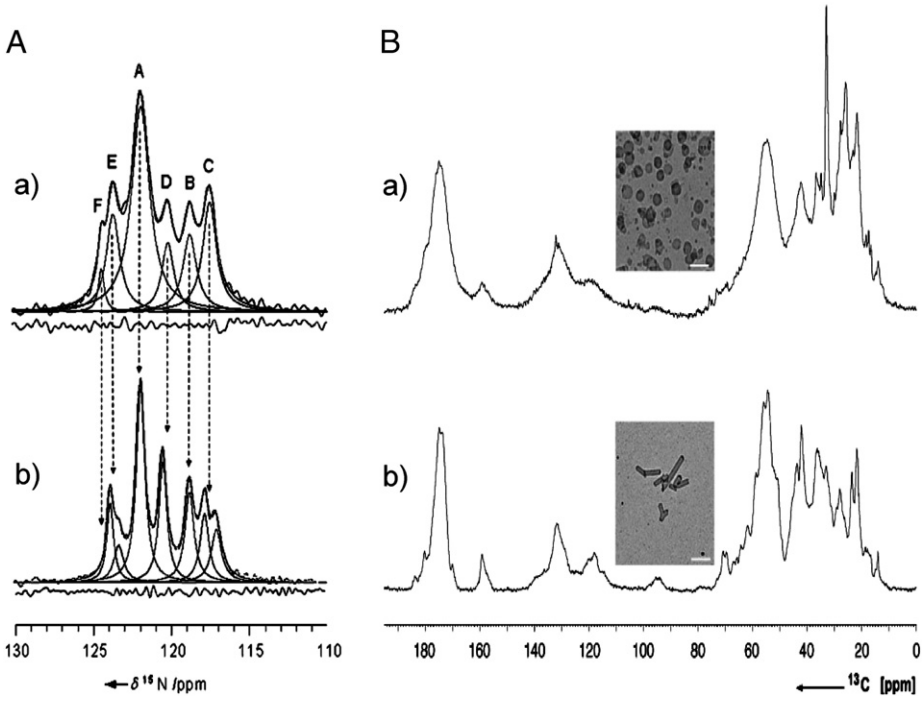

Fig. 7. Effects of sample environments on SSNMR spectra. A: ^{15}N-CP/MAS spectra of ^{15}N-leucine lipid-reconstituted (a) and nanocrystalline (b) DAGK [171]. B: ^{13}C-CP/MAS NMR spectra of ^{13}C, ^{15}N labelled OmpG reconstituted into lipid vesicles (a) and 2D crystals (b). Adapted from [174] with permission from Copyright owners. The spectral resolution of the samples in crystal forms is better than those of the samples in lipid-reconstituted form.

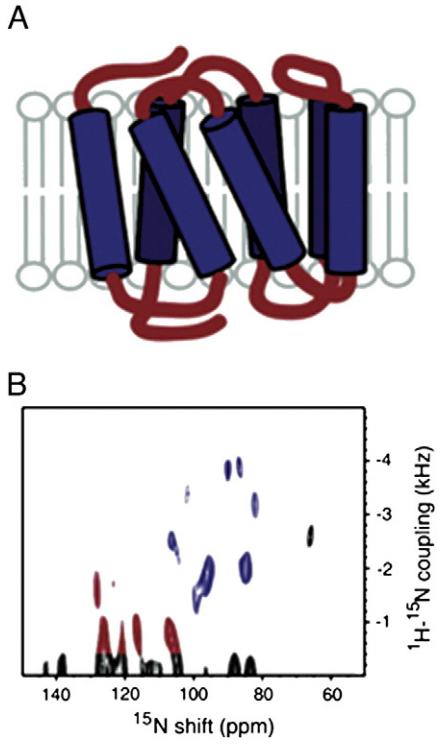

Fig. 8. Structural studies of a GPCR sample in phospholipid bilayers determined by SSNMR spectroscopy. A: CXCR1 model in bilayers. B: 2D PISEMA spectrum of selectively ^{15}N Ile-labelled CXCR1 in magnetically aligned bicelles oriented perpendicular to the direction of the magnetic field. The blue (wheel-like pattern) contours arise from residues in transmembrane helices, whereas red resonances are from residues in loop and terminal regions which are likely to have irregular structures (adapted from [186] with permission from the Copyright owners).

6. Conclusions and future perspectives

GPCR structures are still scarce and their resolution is a major challenge of the membrane structural biology community. Although the origins of the difficulties behind structural studies are becoming clearer, often through laborious and painstaking characterisations, this is by no means routine science.

As in many biophysical methods applied to membrane proteins, expression, biochemical characterisation and labelling (whether Se-Met, with fluorophores or spin labels, or NMR isotopes) provide their own challenges even before the method can be implemented, and in many cases this requires a significant investment of time – often years – which is incompatible with fast returns.

One reason for crystallisation difficulties is the dynamic nature of GPCRs. Since NMR is well suited to define dynamics and ranges of structural constraints, this methodology has the potential to provide new information either *ab initio* or to complement crystal models in which rigid atom structures are defined, although NMR crystallography is now becoming a reality [49,51].

Of specific complementarity is the power of NMR to provide data on ligand binding, either the kinetic or conformational details, which may be less well resolved from other direct or indirect methods. Both solNMR and SSNMR can add this detail for GPCRs [42], but technical hurdles with suitable sample preparation need to be overcome.

In summary, a combined-front approach is essential if we are to bring the power of contemporary biophysics to the GPCR field in the hope of understanding their complexity leading eventually to drug design and then to neurological therapies based on this major class of receptors.

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