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Solution NMR spectroscopy of GPCRs: residue-specific labeling strategies with a focus on ^{13}C -methyl methionine-labeling of the atypical chemokine receptor ACKR3

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Abstract

The past decade has witnessed remarkable progress in the determination of G protein-coupled receptor (GPCR) structures, profoundly expanding our understanding of how GPCRs recognize ligands, undergo activation, and interact with intracellular signaling components. In recent years, numerous studies have used solution Nuclear Magnetic Resonance (NMR) spectroscopy to investigate GPCRs, providing fundamental insights into GPCR conformational changes, allostery, dynamics, and other facets of GPCR function that are challenging to study using other structural techniques. Despite these advantages, NMR-based studies of GPCRs are few relative to the number of published structures, due in-part to the challenges and limitations of NMR for the characterization of large membrane proteins. Several studies have circumvented these challenges using a variety of isotopic labeling strategies, including side chain derivatization and metabolic incorporation of NMR-active nuclei. In this manuscript, we provide an overview of different isotopic labeling strategies, and describe an in-depth protocol for the expression, purification, and NMR studies of the chemokine GPCR Atypical Chemokine Receptor 3 (ACKR3) via $^{13}\text{CH}_3$ -methionine incorporation. The goal of this manuscript is to provide a resource to the GPCR community for those interested in pursuing NMR studies of GPCRs.

Keywords

G protein-coupled receptor; GPCR; solution nuclear magnetic resonance; NMR; methyl labeling; methionine NMR; chemokine receptor; Atypical Chemokine Receptor 3; ACKR3

1. INTRODUCTION

1.1 Overview

Innovations in the expression, purification, crystallization, and microscopy of G protein-coupled receptors (GPCRs) have enabled the determination of more than fifty unique GPCR

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structures to-date, including representative structures from four of the major receptor sub-classes (classes A, B, C, and F), structures of the same receptor with multiple ligands, structures of the same receptor in both inactive and active states, and structures of receptors complexed with the downstream effectors G protein and arrestin (<http://gpcrdb.org>; (Pandy-Szekeres et al., 2018)). In total, these structures have revealed atomic-level insights into the mechanistic basis of GPCR-ligand specificity and GPCR activation. Moreover, the abundance of GPCR structures has enabled computational methods such as molecular dynamics (MD) simulations and molecular modeling to reveal details of GPCR dynamics (Hilger, Masureel, & Kobilka, 2018; Latorraca, Venkatakrishnan, & Dror, 2016), GPCR-ligand/-effector interactions (Koehl et al., 2018; Latorraca et al., 2016; Van Eps et al., 2018; Zheng et al., 2017; Zhou et al., 2017), as well as identify new ligands by virtual screening and structure-based drug design (Manglik et al., 2016; McCorvy et al., 2017).

Owing to many of the same advances in GPCR expression and purification, experimental approaches to studying GPCR dynamics have also made significant progress in the past decade. For instance, NMR, EPR, and fluorescence-based approaches have catalogued conformational and dynamic changes associated with unique GPCR signaling states (i.e., agonist-, antagonist-, inverse agonist-, biased agonist, and effector-bound states) (reviewed in (Hilger et al., 2018; Latorraca et al., 2016)). NMR studies of GPCRs have been particularly informative, reporting on structural changes associated with binding to ligands having variable efficacies (Bokoch et al., 2010; Clark et al., 2017; Eddy, Lee, et al., 2018; Isogai et al., 2016; Kofuku et al., 2012; J. J. Liu, Horst, Katritch, Stevens, & Wuthrich, 2012; Solt et al., 2017; Ye, Van Eps, Zimmer, Ernst, & Scott Prosser, 2016), nanobody binding (Isogai et al., 2016; Manglik et al., 2015; Nygaard et al., 2013; Solt et al., 2017; Sounier et al., 2015; Staus et al., 2016), biased signaling (J. J. Liu et al., 2012; Okude et al., 2015), the effects of physiologic ions (Ye et al., 2018), and even the effect of fusion proteins used for crystallization on GPCR dynamics ((Eddy, Didenko, Stevens, & Wuthrich, 2016); summarized in Table 1). Despite significant progress, NMR studies of GPCRs trail those documenting new receptor structures by a wide margin, with only a few prototypic examples having been studied in detail. Given the fundamental importance of the allosteric basis of signal transduction at GPCRs, more NMR studies of diverse GPCR subtypes will be needed to understand the extent to which the mechanisms of signal transduction are conserved among GPCRs.

NMR approaches to studying GPCRs face many challenges, foremost among which are the size of reconstituted GPCR-membrane mimic systems and the difficulty of isotopic labeling in eukaryotic expression systems. While numerous approaches to the expression and purification of GPCRs in bacterial expression systems have successfully allowed the purification of uniformly labeled receptors for NMR studies (Table 1; (Casiraghi, Damian, Lescop, Baneres, & Catoire, 2018; Casiraghi et al., 2016; Park et al., 2011; Park et al., 2012)), this expression system has limited utility in the absence of GPCR engineering (e.g. thermostabilization techniques), which is technically challenging and in many cases alters the endogenous GPCR signaling properties that may be of interest in NMR-based studies (Vaidehi, Grisshammer, & Tate, 2016).

Despite the challenges of NMR approaches to studying GPCRs, a handful of studies have successfully purified isotopically labeled GPCRs in membrane mimic systems to reveal important insights into GPCR biology (Table 1). In general, the most commonly used approaches use site-specific isotopic labeling of specific amino acid side chains through either chemical modification or metabolic incorporation (Table 1). The chemical modification approach has been used to introduce ^{13}C -methyl groups onto the side chains of exposed lysine residues for two-dimensional experiments, allowing the simultaneous detection of receptor conformational states at multiple sites within the GPCR tertiary structure (Bokoch et al., 2010; Sounier et al., 2015). Alternatively, incorporation of fluorine-containing probes has allowed for the labeling of cysteines either natively occurring at or engineered at the bottom of transmembrane domains 6 and 7, both of which are known to undergo large scale conformational changes during receptor activation (J. J. Liu et al., 2012; Manglik et al., 2015; Ye et al., 2016). In addition to these two strategies, metabolic incorporation of $^{13}\text{CH}_3$ -methionine has become increasingly popular in studies of numerous GPCRs (Casiraghi et al., 2016; Kofuku et al., 2012; Nygaard et al., 2013; Solt et al., 2017).

The goal of this manuscript is to outline a method for the production of GPCRs for solution NMR studies of G protein-coupled receptors. We will define these methods using the chemokine receptor ACKR3, but the methods defined herein are generally applicable for most GPCRs. We will first survey advantages and disadvantages of different strategies to incorporate NMR-sensitive nuclei into GPCRs. We will then focus on incorporation of $^{13}\text{CH}_3$ -methionine, which has become one of the most widely used strategies for solution NMR studies of GPCRs. We will discuss special considerations for purification of NMR-sensitive samples, as well as NMR experimental considerations. Finally, we will discuss strategies for peak assignment to interpret alterations in NMR spectra and correlate these alterations with structurally relevant conformational changes. These methods will serve as a general resource to those who wish to pursue NMR studies on GPCRs.

1.2 Survey of isotopic labeling strategies for NMR studies of GPCRs: sensitivity, cost, natural abundance, and other considerations

With the exception of a few studies (Eddy, Lee, et al., 2018; Isogai et al., 2016), most NMR studies of GPCRs to-date have employed residue-specific side chain labeling strategies with ^{13}C or ^{19}F nuclei that label a single type of amino acid side chain for NMR detection (Table 1). Importantly, each isotopic labeling strategy facilitates the investigation of different components of the of GPCR signal transduction process, so choosing an appropriate labeling method is paramount to investigating a given hypothesis. In this section, we explore the advantages and disadvantages of different NMR nuclei with respect to NMR signal sensitivity, protein production cost, and other considerations.

The most common techniques for studying GPCRs using solution NMR spectroscopy have been (1) 2D NMR using metabolic incorporation of $^{13}\text{CH}_3$ groups at methionine residues, and (2) 1D NMR using cysteine derivatization with ^{19}F -containing probes (Table 1; Figure 1A). These approaches have advantages in cost and sensitivity over conventional ^{15}N -backbone-labeling strategies (Prosser & Kim, 2015), although some groups have achieved

excellent results using fully- (Eddy, Lee, et al., 2018) or selectively- (Isogai et al., 2016) ^{15}N -backbone labeled GPCRs.

Regarding sensitivity, NMR signal sensitivity is proportional to the gyromagnetic ratio of the measured nucleus (i.e., $\text{SNR} = N_T \gamma^{5/2} B^{3/2}$; SNR is signal-to-noise ratio, N_T is total number of spins, γ is the gyromagnetic ratio, and B is the magnetic field; (Goldenberg, 2016)). Given the higher gyromagnetic ratio of ^{13}C versus ^{15}N (^{13}C : $\sim 67 \times 10^6 \text{ s}^{-1}\text{T}^{-1}$; ^{15}N : $\sim 27 \times 10^6 \text{ s}^{-1}\text{T}^{-1}$), ^{13}C labeling boosts sensitivity ~ 10 -fold over ^{15}N labeling for a sample at a given concentration and magnetic field. This sensitivity boost can have significant benefits in the context of GPCR studies, in which sample protein concentration is typically limiting. Moreover, large size of GPCR-micelle complexes translates to more abundant ^1H atoms and slower correlation times, both of which hasten spin relaxation times and decrease sensitivity. The negative effect of abundant ^1H atoms can be minimized by protein deuteration. Deuteration entails the replacement of protein ^1H atoms with less-NMR-active ^2H atoms, which profoundly enhances signal sensitivity in both ^{13}C (Kofuku et al., 2014) and ^{15}N (Eddy, Lee, et al., 2018) NMR studies of GPCRs. Whereas ^{13}C -methyl labeling of GPCRs affords strong signals in the absence of deuteration (discussed below; (Kofuku et al., 2012; Nygaard et al., 2013; Solt et al., 2017)), deuteration in the context of ^{15}N -labeled proteins decreases the sensitivity limitations of the small ^{15}N gyromagnetic ratio ((Eddy, Lee, et al., 2018); Figure 1B). Despite the advantages of deuteration, uniform or residue-selective deuteration adds considerable cost to protein production, particularly in insect cell and mammalian expression systems. Importantly, the higher intrinsic sensitivity of ^{13}C nuclei allow one produce spectra with adequate resolution in the absence of deuteration, giving ^{13}C -labeling a significant advantage over ^{15}N -labeling when one must balance experimental sensitivity and cost (Figure 1B).

Beyond isotope-dependent effects on sensitivity, ^{13}C incorporation by ^{13}C -methyl labeling further enhances signal sensitivity relative to ^{15}N -backbone labeling through two additional mechanisms. Firstly, ^{13}C -methyl groups experience fast, picosecond timescale motions that help to offset relaxation due to slow correlation times of large GPCR-detergent complexes (Kurauskas, Schanda, & Sounier, 2017). Secondly, ^{13}C -methyl groups have three ^1H atoms, providing a three-fold signal enhancement relative to singly protonated heteronuclei in two-dimensional NMR experiments (Kurauskas et al., 2017). In all likelihood, all of these considerations have led to the overwhelming popularity of ^{13}C -methyl labeling for NMR studies of GPCRs.

In comparison to ^{13}C - and ^{15}N -isotopic labeling, ^{19}F -isotopic labeling offers even more sensitivity enhancement for NMR studies for GPCRs. ^{19}F has a gyromagnetic ratio almost as large as ^1H (^{19}F : $\sim 252 \times 10^6 \text{ s}^{-1}\text{T}^{-1}$; ^1H : $\sim 267 \times 10^6 \text{ s}^{-1}\text{T}^{-1}$). In addition, the ^{19}F nucleus gives rise to large chemical shift changes, leading to good peak dispersion between different functional states ((Prosser & Kim, 2015; Ye et al., 2016); Figure 1B).

These labeling strategies are not without drawbacks. One downside to ^{13}C -methyl labeling is the impact of the high natural abundance of ^{13}C on NMR spectra. With a natural abundance of $\sim 1\%$, buffers and detergents, which are included in NMR samples at millimolar concentrations, give strong ^{13}C signals which can obscure peaks corresponding to the probes

of interest (Figure 1B). In comparison, ^{19}F (the NMR-sensitive, naturally abundant fluorine isotope) is absent from proteins and most buffers, affording ^{19}F -based experiments high signal-to-noise ratios. Nevertheless, ^{19}F -labeling strategies used for NMR studies of GPCRs rely upon chemical derivatization of cysteine residues, and these derivatization methods chemically alter residue side chains, changing their properties and potentially altering protein function (Figure 1A). In comparison, metabolic labeling by incorporation of $^{13}\text{CH}_3$ -methionine or other isotopically labeled residues is non-perturbing (Figure 1A).

1.3 Number and distribution of commonly-labeled residues in GPCR structures

The abundance of cysteine, lysine, and methionine residue types varies widely among class A GPCRs. One consideration when choosing a labeling strategy is the number of probes that will be labeled. In principle, more probes have the potential to reveal more structural information by sampling more receptor sites, but in practice more probes can lead to spectral crowding (Bokoch et al., 2010) and increase the burden of peaks for assignment (Eddy, Lee, et al., 2018). To assess the abundance of different residues used for site-specific labeling and NMR studies, we investigated the distribution of these residues among class A GPCRs (Figure 2A). Our analysis reveals an additional advantage of commonly used cysteine, lysine, and methionine labeling strategies: on average, GPCRs have 10–15 of these residues, such that they offer enough probes to sample GPCR structure but are not so abundant as to create problems of spectral crowding or mutagenesis for peak assignment or site-specific labeling (Figure 2A).

A final consideration is that metabolic labeling and derivatization give the user access to different sites in the GPCR tertiary structure, as depicted in Figure 2B. Whereas chemical modification methods label only solvent exposed sites following receptor purification, a distinct advantage of metabolic labeling strategies (including $^{13}\text{CH}_3$ -methionine incorporation) is the ability to label sites in the transmembrane region, providing experimental accessibility to conformational changes of molecular switch residues. Conversely, cysteine and lysine labeling strategies restrict probe access to the intracellular binding domain and orthosteric pocket and extracellular loops (Figure 2B).

Given the popularity and utility of ^{13}C -methionine labeling for NMR studies of GPCRs, this manuscript will outline a method for the incorporation of $^{13}\text{CH}_3$ -methionine into GPCRs made in insect cells (summarized in Figure 3A). We will focus on the production of $^{13}\text{CH}_3$ -methionine-labeled Atypical Chemokine Receptor 3 (ACKR3), but the methods employed herein will be applicable to other GPCRs. We refer the interested reader to excellent papers and reviews on isotopic labeling of GPCRs using other methods, including $^{13}\text{CH}_3$ -lysine labeling (Larda, Bokoch, Evanics, & Prosser, 2012), ^{19}F -cysteine labeling (Prosser & Kim, 2015; Ye, Larda, Frank Li, Manglik, & Prosser, 2015), and uniform labeling in insect cells (Franke et al., 2018).

2. METHODS

2.1 Expression of $^{13}\text{CH}_3$ -methionine labeled GPCRs

2.1.1 Equipment—Heat controlled shaking incubator (must be programmable to maintain a temperature of 27°C)

Benchtop centrifuge (must accommodate Corning, 431123/17410–30)

Thomson Optimum Growth 5L flask (Thomson Instrument Company, 931116)

Corning sterile 500ml conical bottom centrifuge tubes (Corning, 431123)

Corning 431124 rotor cushions (Corning, 17410–30)

Falcon 50ml centrifuge tube (Corning, 352098)

EMD Millipore Steriflip sterile disposable vacuum filter units, 0.22µm (EMD Millipore, SCGP00525)

2.1.2 Reagents—Sf9 insect cells (Expression Systems, 94–001F)

ESF-921 insect cell culture medium (Expression Systems, 96–001-01)

ESF-921 Delta Series methionine deficient insect cell culture medium (Expression Systems, 96–200)

L-methionine (methyl- ^{13}C , 99%) (Cambridge Isotope Laboratories, CLM-206–1)

High-titer ACKR3 baculovirus (see Gustavsson, et al., 2016 (Gustavsson, Zheng, & Handel, 2016) for detailed protocol; please note that we are using the same ACKR3 construct in pFastBac vector described in that paper)

2.1.3 Protocol

1. Grow 2L cells to a density between $2\text{--}4 \times 10^6$ cells/ml in a large volume flask (e.g., Thomson Optimum Growth 5L flask). Cells should be cultured at 27° C in the dark. Shake speeds will vary by shaker, but for 2L of cells in the listed flask growth between 110–130 rpm is suitable. Cells should be split 1–2 days before infection to ensure that the cells reach the desired density on the day of infection. We find that infection of 2L of cells with high titer ACKR3 baculovirus in this density range yields enough protein to run 1–2 NMR experiments under different conditions (i.e., 1–2 samples of ~350ul at 50 µM). Note that infection at higher cell densities (closer to 4×10^6 cells/ml) gives higher yields, but care must be taken to ensure that high enough titer baculovirus is used such that cell growth does not increase above a density of 4×10^6 cells/ml. We find that proteins purified from cell pellets harvested at high densities (i.e., $>4 \times 10^6$ cells/ml) contain more contaminants and have a shorter shelf-life than proteins purified from cells harvested at densities between $2\text{--}4 \times 10^6$ cells/ml.

2. In a sterile hood, pour 2L of cells into four Corning 500ml conical bottom centrifuge tubes. Screw caps tightly to ensure no contaminants enter the flask during centrifugation. Do not dispose of the large volume flask and keep it capped in a sterile hood as it will be reused following cell washes with methionine deficient media.
3. Spin cells for 10 minutes at 800 x g on a benchtop centrifuge. Conical bottom rotor cushions may be required to accommodate the centrifuge tube within the spin bucket.
4. Return the cells to a sterile hood. Aspirate or pour off the media, retaining the pellet.
5. Keeping the cells in the conical bottom flasks, wash the cells with 50–100ml ESF-921 Delta Series methionine deficient media per flask. Resuspend cells using a 50ml serological pipette tip and break up the pelleted cells to ensure that all cells are washed in the methionine deficient media.
6. Spin cells for another 10 minutes at 800 x g on a benchtop centrifuge.
7. Return cells to a sterile hood. Aspirate or pour off the media, retaining the pellet.
8. Resuspend cells in 50–100ml of methionine deficient media, and break up the pelleted cells with a 50ml serological pipette.
9. Transfer cells back into the large volume flask, and top off the methionine deficient media to a final volume of 1L.
10. Infect the cells with high-titer, ACKR3 expressing baculovirus (see Gustavsson, et al., 2016 (Gustavsson et al., 2016) for details on generation of baculovirus and titring).
11. Shake cells at 27°C at 110–130 rpm in a shaking incubator for 5 hours.
12. After 5 hours, return cells to a sterile hood and add 250 mg/L $^{13}\text{CH}_3$ -methionine. $^{13}\text{CH}_3$ -methionine comes in 1g aliquots. Dissolve 1g methionine by adding 20ml milli-Q- H_2O (mqH_2O) to make a 0.05g/ml stock. Shake for ~1h at 37°C to help dissolve the methionine. Sterile filter the dissolved $^{13}\text{CH}_3$ -methionine by transferring it to a 50ml conical, and then filtering using a Steriflip sterile filtering device. For a volume of 2L of cells, add 10ml of $^{13}\text{CH}_3$ -methionine, or 500mg / 2L. Note that we do not adjust the amount of $^{13}\text{CH}_3$ -methionine added by cell density, but have collected equivalent NMR spectra when cells were infected as high as 4×10^6 cells/ml with 250mg/L $^{13}\text{CH}_3$ -methionine. Our 5 hour interim between virus addition and $^{13}\text{CH}_3$ -methionine addition follows Solt, et al., 2017 (Solt et al., 2017), but we note that other papers add $^{13}\text{CH}_3$ -methionine immediately (Nygaard et al., 2013) or wait up to 16 hours to add $^{13}\text{CH}_3$ -methionine (Kofuku et al., 2012). Additionally, at least one protocol adds methionine at a concentration of 200mg/L (Kofuku et al., 2012), all demonstrating the mutability of these parameters within reason. The lag time of $^{13}\text{CH}_3$ -methionine addition is introduced to decrease the amount of $^{13}\text{CH}_3$ -methionine used in the production of non-GPCR proteins in the initial response

to viral infection. This should in turn make $^{13}\text{CH}_3$ -methionine more plentiful during GPCR expression and increase incorporation, although we are unaware of any studies that test the effects of lag time on $^{13}\text{CH}_3$ -methionine incorporation in a GPCR system.

13. At the same time as $^{13}\text{CH}_3$ -methionine is added (i.e., ~5 hours after viral addition), add 1L of methionine-deficient media for a final volume of 2L. Infection at an initially lower volume (1L) followed by dilution 5 hours later (2L) may allow the effective viral concentration to be higher as the virus takes hold, although the cell density is also higher in the smaller volume. We note that other $^{13}\text{CH}_3$ -methionine labeling protocols add virus, $^{13}\text{CH}_3$ -methionine, and the entire volume of methionine deficient media all at once (Sounier et al., 2015).
14. Check the cell density after 24 hours to ensure that the cells are growth arrested (i.e., the cell density should be the same as after the volume was brought to 2L).
15. After 48 hours, measure cell density and viability, and perform a flow cytometry assay using anti-FLAG-FITC antibody as described previously to ensure adequate receptor expression (Gustavsson et al., 2016).
16. Harvest cells by centrifugation at 2000 x g for 15 minutes. Resuspend cells pellets in ice-cold PBS (~50ml / pellet), transfer to 50ml Falcon tubes, re-centrifuge, and store at -80°C until further use.

2.2 Purification and validation of isotopically-labeled GPCRs for NMR studies

2.2.1 Equipment—Amicon Ultra-4 Centrifugal Filter Unit, 30,000 MWCO (Millipore Sigma, UFC803024)

Disposable PD-10 Desalting Column, with Sephadex G-25 resin (GE, 17085101)

Refer to Gustavsson, et al. 2016. *Methods in Enzymology*, sections 2.4 and 2.5 (Gustavsson et al., 2016)

2.2.2 Reagents—Lauryl Maltose Neopentyl Glycol (Anatrace, NG310)

Cholesteryl hemisuccinate (Sigma, C6512)

Refer to Gustavsson, et al. 2016. *Methods in Enzymology*, sections 2.4 and 2.5 (Gustavsson et al., 2016)

2.2.3 Buffers

NMR Exchange Buffer: 25 mM HEPES, pH 7.5

150mM NaCl

0.025%/0.005% MNG/CHS

2.2.4 Protocol

1. Purification of $^{13}\text{CH}_3$ -methionine labeled ACKR3 is similar to the published purification scheme for unlabeled ACKR3 in Gustavsson, 2016, with a few important alterations. As such, we will describe only deviations from that published protocol (Gustavsson et al., 2016). Most of the steps outlined here relating to glycerol, ionic strength, detergent selection, and so forth are not specific to $^{13}\text{CH}_3$ -methionine incorporation or ACKR3 purification but are generally applicable considerations for NMR studies of GPCRs.
2. Small molecule ligands: Small molecule ligands used during receptor solubilization or at any point during the incubation period are added from concentrated stocks (100 mM for the molecule CCX777) dissolved in deuterated DMSO (dDMSO). Dissolving small molecules in dDMSO will reduce natural abundance ^{13}C DMSO peaks that could obscure ACKR3-specific ^{13}C -peaks. When solubilizing ACKR3 into detergent micelles with the small molecule ligand CCX777, we add the molecule to a final concentration of 50 μM .
3. Glycerol: Following cobalt-affinity chromatography, Gustavsson, et al. 2016 exchange their protein into “Exchange Buffer” containing 10% glycerol (Gustavsson et al., 2016). While glycerol may enhance receptor stability, the inclusion of glycerol will give rise to peaks in a 2D ^{13}C -HSQC NMR experiments (due to ^{13}C natural abundance) that can obscure $^{13}\text{CH}_3$ -methionine peaks. To avoid the introduction of glycerol peaks in NMR spectra of ACKR3, we substitute the “Exchange Buffer” from Gustavsson, 2016, for “NMR Exchange Buffer,” consisting of 25mM HEPES, 150mM NaCl, and 0.025%/0.005% MNG/CHS (Gustavsson et al., 2016). Users must also be careful to avoid the inadvertent introduction of glycerol during concentration. Benchtop spin concentrator filters (e.g., Amicon Ultra-4 listed above) contain trace amounts of glycerol in the concentration filter and must be washed extensively to avoid glycerol introduction. Finally, Gustavsson, et al., 2016, describes concentrating the ACKR3 sample after cleavage with PNGaseF and Prescission Protease (see section 2.4.4 of Gustavsson, et al. 2016; (Gustavsson et al., 2016)). These enzymes are dissolved in a glycerol-containing buffer, so simply concentrating the sample after cleavage and reverse cobalt steps will not get rid of the glycerol. After PNGaseF/Prescission Protease cleavage, incubation with cobalt, and collection of receptor-containing flow-through, we concentrate the sample and desalt into NMR Exchange Buffer using a PD-10 desalting column to ensure removal of glycerol introduced with addition of enzymes. The sample is subsequently concentrated to a stock of 300–400ul before preparing an NMR sample.
4. Ionic strength: The “Exchange Buffer” described in Gustavsson, et al., 2016, contains 400 mM NaCl (Gustavsson et al., 2016). High salt concentrations (e.g., > 200mM NaCl) decrease signal-to-noise of cryoprobe-collected data by increasing sample conductivity (Voehler, Collier, Young, Stone, & Germann, 2006). Additionally, high salt samples increase 90° (i.e., P1) pulse widths,

particularly in 5mm NMR tubes (Voehler et al., 2006). To decrease the negative effects of high salt concentrations on NMR data acquisition, we have adjusted Gustavsson, et al.'s Exchange Buffer to include 150mM NaCl instead of 400mM NaCl.

5. Detergent micelles: We have substituted use DDM/CHS mixed micelles used in Gustavsson, et al., 2016, for LMNG/CHS mixed micelles, following the popularity of this detergent in numerous recent publications (Table 1; (Eddy, Lee, et al., 2018; Korczynska et al., 2018; Manglik et al., 2015; Solt et al., 2017; Soumier et al., 2015; Ye et al., 2016)). We find increased sample shelf-life in LMNG/CHS mixed micelles as compared to DDM/CHS mixed micelles, with ACKR3 sample stability for up to 1 month. LMNG/CHS is substituted at all points in Gustavsson, et al.'s protocol (i.e., in Solubilization, Wash 1, Wash 2, and Elution buffers) at the same final concentration, although LMNG/CHS stocks must be made at 5% LMNG and 1% CHS instead of 10% DDM and 2% CHS due to solubility limitations.
6. Note on nanodiscs: ^{13}C -methyl-labeled NMR studies of GPCRs have been performed in both detergent micelles and nanodiscs. While nanodiscs have become increasingly popular due to their advantageous effects on receptor stability and their native-like environment (Table 1; (Kofuku et al., 2014; Okude et al., 2015; Shiraishi et al., 2018)), nanodiscs present some challenges for NMR studies. Nanodiscs are much larger than detergent micelles and thus decrease the correlation time of encapsulated proteins, which decreases T_2 relaxation times and broadens linewidths, causing crowded spectra and obscuring peaks and chemical shift perturbations. The NMR studies of GPCRs that have used nanodiscs to study ^{13}C -methyl-labeled proteins have used deuterated protein (Table 1), which counteracts decreases in T_2 relaxation times by decreasing contributions of dipole-dipole relaxation from ^1H atoms. We have not experimented with nanodiscs, but caution interested users that deuteration may be necessary to get adequate resolution in a nanodisc system. We note that some studies have developed smaller nanodiscs with more favorable properties for NMR studies (Hagn, Etzkorn, Raschle, & Wagner, 2013; Hagn, Nasr, & Wagner, 2018).
7. Validation of receptor purity and stability: Despite incorporation of $^{13}\text{CH}_3$ -methionine and alterations in sample conditions, we are able to isolate pure, monodisperse ACKR3 samples with equivalent thermostabilities as those described previously (Figure 3B).

2.3 NMR studies of $^{13}\text{CH}_3$ -methionine-labeled GPCRs

2.3.1 Equipment—Shigemi 5mm D₂O susceptibility matched microtube (Shigemi, BMS-005TB)

Thin-stem transfer pipette (Cole-Parmer, UX-06226–65)

Bruker Avance 800MHz spectrometer with $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ TCI cryoprobe (Bruker)

2.3.2 Reagents—Purified ACKR3 in detergent micelle

D₂O (99.9%; Sigma, 151882)

2.3.3 Preparing an NMR sample

1. Using a purified protein stock, make an NMR sample with a final volume between 310–400 μ l, a final concentration between 35–100 μ M by diluting in NMR Exchange Buffer (20mM HEPES, pH 7.5, 150mM NaCl, 0.05%/0.0025 MNG/CHS), and 10% D₂O by volume. Attempts to collect spectra below receptor concentrations below 30 μ M have produced inadequately noisy spectra in our system, but some publications have reported using 20 μ M (Sounier et al., 2015) and even as little as 5 μ M protein in some contexts (Kofuku et al., 2012).
2. Note on NMR tube selection: We use a Shigemi 5mm D₂O susceptibility matched microtube. Other experimenters have used 3mm tubes (Casiraghi et al., 2016), which require lower sample volumes and have resolution advantages in high-salt conditions (Voehler et al., 2006), however 3 mm tubes experience a modest decrease in signal intensity under standard conditions since less sample is contained in the probe coil.
3. Transfer the prepared NMR sample to an NMR tube. Use a sample depth gauge to adjust the tube position in the spinner to ensure the sample will be centered in the probe. Load the NMR sample in the magnet.

2.3.4 Running a 2D ¹H-¹³C-HSQC

1. Since software and protocols for running NMR experiments will be unique to each NMR facility, we will focus on general experimental details in this section and highlight those details with particular relevance to ¹³CH₃ NMR studies of GPCRs.
2. Load the sample in the magnet, then adjust the deuterium lock, tune/match ¹H and ¹³C nuclei, shim, calibrate the ¹H P1 pulse, and perform receiver gain adjustment. More details on spectrometer and sample setup can be found in the Bruker Topspin manual (Bruker, 2016) or corresponding manuals for Varian or other spectrometer manufacturers.
3. Setup a two-dimensional HSQC experiment by copying an experimental directory of a ¹H-¹³C-HSQC from a user directory or a pulse program library. The data collected in this manuscript are collected with a standard ¹H-¹³C-HSQC.
4. Set the centering frequency of the experiment to 20 ppm in ¹³C and 4.78 ppm for ¹H (centered at the H₂O). The ¹³C-methyl region for the ϵ -methyl of methionine is centered at ~ 17 ppm in ¹³C and 2 ppm in ¹H in most previous NMR studies of ¹³CH₃-methionine labeled GPCRs (Supp. Table 1), although there is some variation in peak positions in the ¹³C-dimension (e.g., ¹³C-methionine peaks in Casiraghi, et al. 2015 are centered at 2 ppm in ¹H and 14 ppm in ¹³C; (Casiraghi et al., 2016)).

5. Set the spectral width to 16,666.7 Hz in ^1H and 6666.7 Hz in ^{13}C , corresponding to ~ 20 ppm in ^1H and ~ 33 ppm in ^{13}C (spectral width in ppm = spectral width in Hz / base frequency for a given nuclei in Hz/ppm). We note that this window is larger than is required to identify $^{13}\text{CH}_3$ -methionine peaks that located at $\sim 2 \pm 1$ ppm in ^1H and 17 ± 3 ppm in ^{13}C , but we chose a larger window to see the full range of ^{13}C peaks arising from labeled methionine as well as detergents, buffers, and natural abundance ^{13}C protein labeling. Narrowing the spectral width as in other published studies (e.g., 16 ppm in ^1H x 24 ppm in ^{13}C from Kofuku, 2012, Supp. Table 1; (Kofuku et al., 2012)) will decrease data collection time, but care must be taken to avoid folding detergent or other peaks into $^{13}\text{CH}_3$ peaks of interest.
6. Set the number of complex points in the ^1H and ^{13}C dimensions. We are collecting 1024×180 points for ^1H and ^{13}C , respectively, although we note some variation in the number of points used in previous studies (Supp. Table 1).
7. Set the number of scans. Early studies should collect data with a high number of scans (e.g., 256 or 512) to optimize resolution and experimental times. Signal increases on the order of $(\text{number of scans})^{1/2}$, such that increasing the number of scans from 128 to 512 will double the amount of signal collected. Most published studies collect 256 scans for experimental times on the order of 5–12 h (Table 1). Since we do not experience sample stability limitations in the order of days, we routinely collect 512 scans for an experimental time of 27 h to maximize sensitivity.

2.3.5 Additional considerations for 2D experiments on $^{13}\text{CH}_3$ -methionine labeled GPCRs

8. Spectrometer field: Most experiments on ^{13}C -methyl labeled GPCRs have been performed at fields of 800MHz or higher (Table 1) to counteract unfavorable relaxation properties due to the size of GPCR-micelle (or -nanodisc) systems as well as challenges associated with production of high concentration GPCR samples.
9. HSQC versus HMQC: While we and others use standard HSQCs (Bokoch et al., 2010; Korczynska et al., 2018; Nygaard et al., 2013), numerous studies use ^{13}C -HMQC pulse sequences for studies of $^{13}\text{CH}_3$ -labeled GPCRs (Supp. Table 1). HMQCs have been shown to have a TROSY-like effect on peak resolution for ^{13}C -methyl groups, however this effect is only seen in the context of perdeuterated protein (Tugarinov, Hwang, Ollerenshaw, & Kay, 2003). Nevertheless, HMQC pulse sequences contain fewer 90° pulses, which may help preserve signal via less manipulation of bulk magnetization through the course of an experiment (Mandal & Majumdar, 2004). A number of alterations to pulse programs have been implemented for ^{13}C -studies of GPCRs to decrease data collection time (e.g., SOFAST experiments and non-uniform sampling, NUS) and increase resolution (e.g., echo-/anti-echo gradient coherence selection; Supp. Table 1). These alterations can be advantageous when spectrometer access and sample stability are limiting.

10. Mutagenesis to reduce spectral crowding: Numerous studies of ^{13}C -GPCRs have performed extensive mutagenesis of existing methionines to reduce spectral crowding and focus on a subset of methionines of interest. After preliminary testing, users should consider peak overlap and whether mutagenesis of individual methionines may facilitate peak assignment and interpretation of chemical shift perturbations.

2.4 Analysis and interpretation of $^{13}\text{CH}_3$ -methionine-labeled GPCR NMR data

2.4.1. Software—Bruker Topspin software (Bruker)

NMRPipe (Delaglio et al., 1995)

Xeasy (Bartels, Xia, Billeter, Guntert, & Wuthrich, 1995)

2.4.2 Data processing

1. Convert FID time domain data to NMRpipe format using NMRpipe conversion scripts. In NMRpipe, the [fid.com](https://spin.niddk.nih.gov/NMRPipe/doc1/#NMRPIPE%20WORKFLOW) script converts data from Bruker format to a generalized format that can be processed by NMRpipe software. Check that the centering frequencies are properly indexed. For instance, the ^1H signal center position is indexed as 4.773 ppm, so at a temperature of 298 K adjust this to 4.78 ppm. Additional fields may need to be adjusted with more complex data acquisition schemes (see <https://spin.niddk.nih.gov/NMRPipe/doc1/#NMRPIPE%20WORKFLOW>). Once all fields have been verified, and the script has been saved, run the script to convert NMR data to NMRpipe format. The [fid.com](https://spin.niddk.nih.gov/NMRPipe/doc1/#NMRPIPE%20WORKFLOW) generates a **test.fid** file, which can be read by NMRDraw. More details can be found at <https://www.ibbr.umd.edu/nmrpipe/index.html>.
2. Identify optimal peak phasing using NMRDraw. Load data using NMRDraw, and phase the spectrum by adjusting the zero-order (i.e., P0) phasing. Phasing should be adjusted such that the signals of interest (centered at ~2ppm in ^1H) are phased positively and in absorption mode. Record the P0 phasing value.
3. Run an NMRpipe “pipe script” to process the 2D dataset. The NMR pipe script will read the **test.fid** file apply additional processing functions to the data including baseline correction (**POLY -auto**), solvent suppression (**POLY -time**), phase correction (**PS**), apodization (**SP**), zero-filling (**ZF**), and fourier transform (**FT**). These processing functions can be edited in the NMRpipe script using the NMRDraw GUI or manually in a text editor (e.g., vi editor in UNIX). The flags and values associated with these functions must be optimized for each system. The P0 phasing must be manually adjusted for each experiment in the pipe script using the value identified in step 2. The pipe script will generate processed data **test.ft2** file. We use an additional command using xyza2pipe software (<https://github.com/yokochi47/xyza2pipe>) to convert the data to a **test.3D.param** file, which can be read by Xeasy (Yokochi, 2017).

4. Plot the data using Xeasy or other plotting software. Alter the contour, zoom into peaks of interest, and plot the data to save in a format that can be used in an illustration software such as Adobe Illustrator or Inkscape.
5. Identify peaks corresponding to $^{13}\text{CH}_3$ -methionine labeled protein. As discussed in previous sections, $^{13}\text{CH}_3$ -methionine peaks should be centered at $\sim 2 \pm 1$ ppm in ^1H and 17 ± 3 ppm in ^{13}C . While this region is the known location of $^{13}\text{CH}_3$ -methionine methyl groups in random coil elements, care must be taken to identify which peaks actually correspond to peaks from $^{13}\text{CH}_3$ -methionine (versus detergent or buffer components) and how widely these peaks are dispersed from the approximate center by assignment and carefully tracking chemical shift perturbations.
6. The construct of ACKR3 used in this study has 8 methionines (Gustavsson et al., 2016). Shown in Figure 4A are 9 distinct peaks, each of which may correspond to one of these labeled methionines (although as discussed in subsequent sections, some peaks likely correspond to non-protein, buffer or detergent components).

2.4.3 Peak volume quantification

1. In this section we provide instructions for peak volume quantification using the NMRdraw Nonlinear Spectral Lineshape Modeling (nlinLS) functionality. Please note that steps detailed here are adapted from NMRpipe (<https://spin.niddk.nih.gov/NMRPipe/doc2new/#PEAK%20DETECTION%20QUICK%20HOW-TO>), an online guide from Justin Douglas, PhD, at the University of Kansas (<https://nmr.ku.edu/sites/nmrlab.ku.edu/files/docs/LineshapeFitting.pdf>), and Sounier, et al. 2015 (Cornilescu; Douglas, 2012; Sounier et al., 2015).
2. Open data in NMRDraw, zoom to region of interest, and adjust contours. Open the processed NMR spectrum (i.e., **test.ft2** file) in NMRDraw and adjust the contour levels such that peak intensities are represented fully with minimal inclusion of peak tails and noise. Zoom into the region of interest using the 2D zoom function under the “Mouse” tab.
3. Perform peak picking and save to a peak file. Load the “Peak Detection” window under the “Peak” tab. First pick the peaks of interest by choosing “INDEX” as the “Labels” variable and clicking “Detect.” This button will generate a **peak.tcl** script in the current folder, which will identify and number peaks in the zoomed region at peak centers. Peak positions can be adjusted using the “Edit” button (more details at the NMRDraw webpage). Save peak positions by choosing “Save.” Peaks will be written to the default **test.tab** filename.
4. Perform cluster picking and save to a file. Change the “Labels” variable to “CLUSTID” and choose “Draw.” This function will relabel peaks based on which peaks belong to shared and independent peak clusters. Peak cluster designations can be altered using the “edit” button as before. Save cluster IDs by choosing “Save.”

5. Calculate peak volumes for each peak. In order for peak volumes to be calculated for peaks belonging to clusters of overlapping peaks, peak shapes must be interpolated for every peak such that the volume estimation calculates volumes for each peak as opposed to the entire peak cluster volume. NMRDraw utilizes a script called **autoFit.tcl** which simulates a spectrum based on the given peak positions and cluster ID's in which each peak is represented independently. In a separate Unix command line shell, navigate to the experimental directory and run the command **autoFit.tcl -specName test.ft2**, which will generate **autofit.com**, **aux.tab**, **diff.ft2**, **nlin.tab**, and **sim.ft2** files. Note that in this command **test.ft2** is the name of the 2D NMR spectrum.
6. Inspect the **sim.ft2** and **diff.ft2** files. The file **sim.ft2** is the simulated 2D spectrum and the **diff.ft2** file is the difference spectrum of the actual data (**test.ft2**) and the simulated data (**sim.ft2**). Open these files in NMRDraw and ensure that the simulated spectrum accurately represents the actual data, and that all peaks of interest disappear in the difference spectrum. If the difference spectrum shows no peaks in the region of interest, the simulated spectrum appropriately predicted the peak volumes and is suitable for peak volume analysis.
7. The peak volumes are written to the file **nlin.tab**. The columns of interest are the INDEX column, which refers to the chosen peaks, and the VOL column, which lists peak volumes. More information on additional peak attributes can be found on the NMRDraw webpage listed above.
8. Open the peak table in a plotting software such as R to graph peak volumes. Typically, peak volumes are normalized to a single peak, often one that is invariant under different conditions (Nygaard et al., 2013).

2.4.4 Interpretation of 2D NMR data

1. Interpreting conformational changes from chemical shift perturbations: Chemical shift perturbations (CSPs) reflect changes in the local chemical environment of a given probe. CSPs result from direct interactions with added perturbants (e.g., ligand, pH, salt, etc.) or conformational changes induced by perturbant binding at a distant site. In the context of GPCRs, numerous NMR studies have documented peaks that do (and do not) undergo CSPs in the context of ligands with different efficacies (i.e., inverse agonists, antagonists, partial agonists, biased agonists, and full agonists). By documenting these changes by NMR, these studies identify the specific domains that contribute to (or are unimportant for) direct ligand interactions or transmitting conformational changes across the GPCR structure. For instance, in a recent example, Solt and colleagues performed NMR studies with ¹³CH₃-methionine-labeled β_1 -adrenergic receptor (β_1 AR) with numerous ligands, and identified CSPs in probes positioned at the intracellular domains of TMs 5 and 6 that linearly correlate with ligand efficacy (Solt et al., 2017). By documenting these changes, the authors confirm the importance of conformational changes in TMs 5 and 6 for receptor activation.

2. Interpreting conformational populations from peak intensities: Previous studies have used peak volume as a proxy for receptor conformational exchange at the peak-containing domain. Nygaard and colleagues measured decreases in peak volumes of peaks corresponding to TMs 5 and 6 for agonist-bound β_2 AR as compared to antagonist bound β_2 AR, suggesting that agonists induce a destabilization of these domains. Subsequent addition of an intracellular-binding nanobody increased peak volumes at TMs 5 and 6 beyond their volumes in the inactive state, suggesting that the nanobody conferred increased receptor stability in these domains through direct interactions (Nygaard et al., 2013). Similar outcomes were reported in analogous studies of the μ -opioid receptor (μ OR) (Sounier et al., 2015) and β_1 AR (Solt et al., 2017). Solt and colleagues attribute broadening of $^{13}\text{CH}_3$ peak intensities to conformational exchange on the millisecond-microsecond (ms- μ s) timescale, with less-intense peaks demonstrating faster exchange (Solt et al., 2017). In the context of ACKR3, we see peaks 6–8 accounting for the largest peak volumes, suggesting they undergo the least conformational exchange on the ms- μ s timescale, and peaks 1–5 and 9 with the smallest volumes, likely more dynamic on the same timescale (Figure 4B). Analysis of peak volume changes in under different conditions (e.g., with different ligands) in future studies will allow us to interpret the effects of these conditions on ms- μ s conformational exchange at labeled sites.
3. Interpreting methionine χ_3 dihedral angles from chemical shift values: ^{13}C -methyl shifts can be used to discriminate *gauche* and *trans* conformations of the methionine methyl χ_3 dihedral angle (Butterfoss et al., 2010; Kofuku et al., 2012; London, Wingad, & Mueller, 2008; Solt et al., 2017). Specifically, chemical shifts in the range of ~15.8–16.8 p.p.m. ppm correspond to *gauche* orientations ($\sim \pm 67^\circ$), whereas shifts in the ~18.4–19.5 p.p.m. region correspond to a *trans* orientation ($\sim 180^\circ$; (Butterfoss et al., 2010; London et al., 2008)). In ACKR3, we see that peaks 1 and 2 fall within the *gauche* region of the spectrum, whereas peak 9 falls within the *trans* region of the spectrum (Figure 4C).
4. Interpreting methionine-aromatic residue interactions from chemical shift values: Upfield ^1H shifts relative to methionine random coil values of ~2.1 p.p.m. can be interpreted as proximity to aromatic side chains due to so-called “ring current shift” effects (D. Liu & Wuthrich, 2016). ACKR3 peaks 1–4 demonstrate this property, suggesting close proximity of aromatic side chains to these residues (Figure 4C).

2.5 NMR peak assignment

2.5.1 Equipment—PCR thermocycler

2.5.2 Reagents—QuickChange II Site Directed Mutagenesis Kit (Agilent, 200521)

2.5.3 Software—GPCRdb website (<http://www.gpcrdb.org>; (Pandy-Szekeres et al., 2018))

QuickChange Primer Design website (<https://www.genomics.agilent.com/primerDesignProgram.jsp>)

2.5.4 Protocol

1. Identification of suitable methionine substitutions for mutagenesis: Peak assignment of $^{13}\text{CH}_3$ -methionines is performed by single residue amino acid mutagenesis. We were concerned that some residues may be challenging to assign due to spectral overlap in the main cluster of $^{13}\text{CH}_3$ -methionine peaks (Figure 4A), so we chose to create a methionine-less ACKR3 mutant (ACKR3 Met) and individually reintroduce methionine residues. To identify suitable methionine substitutions for our ACKR3 Met mutant, we performed a sequence alignment of ACKR3 with all chemokine receptors using GPCRdb (Pandy-Szekeres et al., 2018). We identified the most commonly occurring residue at the structurally equivalent position and used this to guide mutations, although in some cases mutated ACKR3 to match CXCR4 at the equivalent position, as ACKR3 and CXCR4 share the chemokine ligand CXCL12. Since N-termini lack structure-based alignments in GPCRdb, we chose to mutate M33 and M37 to alanine with the rationale that these constitute minimally perturbing mutations. Our resulting ACKR3 Met construct contains the following mutations: M33A, M37A, M59I, M112I, M138I, M159I, M212L, M327L.
2. For each substitution, design primers using the QuickChange primer design website, and using designed mutagenesis primers follow the protocol listed in the QuickChange II Site Directed Mutagenesis (SDM) Kit to perform mutagenesis. We ordered an ACKR3 Met mutant and cloned it into our pFastBac1 construct. To assign methionines, we reintroduce native methionines into our ACKR3 Met construct using SDM. Specifically, we have mutated reverted L138 to its original methionine in the ACKR3 Met construct (ACKR3 Met L138M).
3. Make high titer baculovirus following Gustavsson, et al., 2016 (Gustavsson et al., 2016), and express, purify, and validate $^{13}\text{CH}_3$ -methionine labeled ACKR3 mutants as described in sections 2.2–2.4 of this manuscript.
4. Perform NMR experiments with mutated ACKR3 and make overlays of NMR spectra. In this study, we made three constructs in total, all grown in methionine deficient media supplemented with $^{13}\text{CH}_3$ -methionine: (1) WT-ACKR3, as described above (Figure 4A), (2) ACKR3 Met (Figure 5A), and (3) ACKR3 Met L138M (Figure 5B). To make peak assignments, identify peaks that disappear in the spectrum of the ACKR3 mutant spectrum as compared to the WT-ACKR3 spectrum. Comparison of the WT-ACKR3 spectrum to ACKR3 Met shows that peaks 1, 2, 4, 5, part of peak 6 (referred to as peak 6b), 7, 8, and 9 disappear with mutation of ACKR3 methionines, identifying the correspondence between each of these peaks and the $^{13}\text{CH}_3$ -labeled methionine introduced in the receptor. We next collected an NMR spectrum of ACKR3 Met M138 grown with $^{13}\text{CH}_3$ -methionine. When overlaid with WT-ACKR3 peak 1

reappears, supporting the identity of this peak as M138 in the WT-ACKR3 sequence (Figure 5B).

3. SUMMARY AND CONCLUSIONS

Solution NMR studies of GPCRs have provided fundamental insights into the mechanisms of GPCR signaling by revealing novel insights into receptor conformations and dynamics. Site-specific side-chain labeling strategies have predominated NMR efforts to study GPCRs, and $^{13}\text{CH}_3$ -methionine labeling is becoming one of the most utilized isotopic labeling strategies to interrogate the mechanistic details of GPCR signaling by virtue of $^{13}\text{CH}_3$ -methionines being non-perturbing, the availability of methionine deficient media, the advantageous relaxation properties of $^{13}\text{CH}_3$ groups for NMR studies of large protein complexes, and the ability of this labeling strategy to incorporate probes spanning the lipid bilayer. The method described here should be generally applicable to other GPCRs – indeed, our approach is similar to that used in many published papers (summarized in Table 1 and Suppl. Table 1). Nevertheless, each GPCR will require specific adjustments to the protocol defined through trial and error. Our hope is to provide a starting point for those interested in pursuing NMR studies of GPCRs, as well as provide some context and rationale for choosing $^{13}\text{CH}_3$ -labeling strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison of different isotopic labeling approaches for solution NMR studies of GPCRs.

(A) Chemical structures of natural and modified residues used in NMR studies of GPCRs with isotopic labels indicated by colored circles and derivatized group indicated with colored bonds. (B) Comparison of ¹³C, ¹⁹F, and ¹⁵N isotope features in the context of NMR studies of GPCRs. * We designate deuteration as “advisable” for ¹⁵N labeling due to drastic improvements seen with deuteration in Eddy, et al., 2018 (Eddy, Lee, et al., 2018), although we note that Isogai, et al. 2015., perform selective ¹⁵N-Val backbone labeling with excellent results in the absence of deuteration (Isogai et al., 2016). Note that a few studies have seen improvement in ¹³C NMR studies of GPCRs with deuteration (Clark et al., 2017; Kofuku et al., 2014; Kofuku et al., 2018), but a majority of ¹³C studies are performed without

deuteration with excellent peak resolution (Kofuku et al., 2012; Nygaard et al., 2013; Solt et al., 2017; Sounier et al., 2015).

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Figure 2. Distribution of residues commonly used for isotopic labeling and NMR studies among class A GPCRs.

(A) Distributions of different residue types among class A GPCRs. Sequences of class A GPCRs were obtained from GPCRdb (<http://gpcrdb.org>; (Pandy-Szekeres et al., 2018)) and analyzed for the distribution of residue numbers for Ala, Cys, Gly, Ile, Lys, Met, and Val. Cys. Means are shown within each “violin” as diamonds. Among residues that have been used for isotopic labeling (Table 1), Met, Cys, and Lys (highlighted in gray) are represented ~10–15 times in most class A GPCR sequences, such that selection of any of these residues for specific labeling should yield a small but manageable number of residues. Other probes (e.g., Ala and Val) are represented ~27 on average in a GPCR, such that labeling these will yield crowded spectra in the absence of extensive mutagenesis, particularly in $^{13}\text{CH}_3$ experiments where chemical shift ranges are relatively narrow. (B) Probe distribution for different labeling strategies used in NMR studies of GPCRs. Probes that were labeled are represented as spheres with the labeled regions shaded. Whereas Cys and Lys residues are excluded to cytosolic-facing sites, Met labeling is the only of these three labeling strategies that has access to the transmembrane region. PDB IDs 3SN6 (β_2 AR), 5C1M (μ OR), 5G53 (A_{2A}).

Figure 3. Purification scheme for isotopically labeled GPCRs and validation of $^{13}\text{CH}_3$ -methionine-labeled ACKR3 purity and thermostability.

(A) Purification scheme of isotopically-labeled GPCRs for insect cell system. Insect cells are infected with high-titer baculovirus and harvested 48h later. Cells are grown with $^{13}\text{CH}_3$ -methionine in methionine-deficient media. Receptors are solubilized by incubating cell pellets with detergent (LMNG/CHS micelles) in the presence of ligand. Detergent-solubilized receptor is purified using a cobalt column, followed by tag removal, deglycosylation, and buffer exchange into NMR-suitable buffer. The data presented in (B) and (C) reflect $^{13}\text{CH}_3$ -methionine-labeled ACKR3, but alternatives for isotopic labeling are also depicted. For instance, isotopic labels can be incorporated after affinity column purification by chemical derivatization methods to yield cysteine- or lysine-labeled GPCRs. (B) Representative SDS-PAGE gel stained with Coomassie gel showing pre-cut WT-ACKR3-CCX777 complex, C-terminal FLAG-10x-His cut ACKR3-CCX777, and the final deglycosylated ACKR3-CCX777 sample used for NMR studies. (C) Size exclusion chromatography trace demonstrates a pure, monodisperse ACKR3-CCX777 sample for NMR studies. The sample was run on a Superdex S200 10/300GL Increase column at a flow rate of 0.5ml/min. The column was run using NMR Exchange Buffer, described in Section 2.3.3. (D) CPM melting assay of $^{13}\text{CH}_3$ -methionine labeled WT-ACKR3-CCX777 gives a T_m consistent with previously published studies (Gustavsson et al., 2016). See Gustavsson, 2016 for experimental details.

Figure 4. NMR studies of ACKR3 in detergent micelles and analysis of peak intensity and position.

(A) ^1H - ^{13}C -HSQC of $^{13}\text{CH}_3$ -methionine labeled ACKR3. Positive peaks (solid contours) putatively corresponding to labeled methionines are numbered from 1–9. The construct utilized for these experiments contains 8 methionines. Negative peaks (dashed contours) correspond to natural abundance buffer or detergent signals aliased from outside the ^{13}C spectral window. (B) Analysis of relative peak volumes. Peaks were normalized relative to peak 7. (C) ^{13}C - ϵ chemical shift values encode information about methionine χ_3 angles (*left, middle*) (Butterfoss et al., 2010; London et al., 2008). *Left*: chemical shift values in the shaded ~15.8–16.8 p.p.m. region correlate with *gauche* conformations ($\sim \pm 67^\circ$). The appearance of peaks 1 and 2 in this region suggests that they correspond to a methionine in *gauche* conformation. *Middle*: chemical shift values in the shaded 18.4–19.5 p.p.m. region correlate with a *trans* conformations ($\sim 180^\circ$). The appearance of peak 9 in this region suggests that it corresponds to a methionine in *trans* conformation. *Right*: ^1H chemical shift values encode information about probe proximity to aromatic sidechains. Upfield deviations from ~2.1 p.p.m. in the ^1H dimension indicate close proximity to aromatic side chains

(Kofuku et al., 2012; D. Liu & Wuthrich, 2016). The appearance of peaks 1–4 in this region suggests that these peaks correspond to methionines experiencing ring-current shifts. *Left* and *middle* panels were inspired by (Solt et al., 2017).

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Figure 5. Peak assignment of residue Met138 using ACKR3 Methionine reintroduction construct.

(A) Overlay of WT-ACKR3 (blue) with ACKR3 Δ Met construct lacking all methionines. Peaks that disappear are labeled in blue and correspond to signals from the 8 methionines in the ACKR3 construct. Residual peaks 3 and 6a likely correspond to buffer and detergent signal. Peak 6 partially disappears (i.e., 6a), however some portion of that peak remains (i.e., 6b), possibly corresponding to a labeled methionine peak that is obscured by peak 6a. (B) Overlay of WT-ACKR3 (blue) with ACKR3 Δ Met construct with Met138 reintroduced (i.e., ACKR3 Δ Met I138M). As denoted by the red asterisks, one major peak reappears with reintroduction of Met138, suggesting that peak 1 corresponds to residue Met138. Note that two additional peaks (peaks 4 and 5) show some intensity with Met138 reintroduction that may correspond to alternate states of Met138. (C) Zoomed in region from (A), *top*, and (B), *bottom*. Overlays of WT-ACKR3 spectrum and ACKR3 Δ Met (top) or ACKR3 Δ Met I138M (bottom) shows reappearance of a peak at approximately 1.30 ppm (1 H) x 16 ppm (13 C), supporting assignment of this peak as residue 138.

Table 1.
Summary of isotopic labels used in solution NMR studies of G protein-coupled receptors.

This table summarizes solution NMR studies of GPCRs organized alphabetically by the name of the amino acid labeled (or, in a few cases, uniform ^{15}N labeling designated as “All/backbone”). Also listed are the receptor expression system, the type of NMR experiment, the nuclei detected, the approximate number of assigned peaks, whether the protein (or parts of the protein: e.g., methionine side chain protons) was deuterated, the detergent or membrane mimic used, and the spectrometer field strength. Some studies are listed multiple times because they use multiple labeling strategies (Park et al., 2011), or use a single labeling strategy (i.e., uniform ^{15}N labeling) to interpret labels of different classes (i.e., tryptophan indole ^1H - ^{15}N and backbone ^1H - ^{15}N ; (Eddy, Gao, et al., 2018)). We emphasize that this table is intended as a survey of labeling techniques used in solution NMR studies of GPCRs and is not comprehensive.

Labeled Residue	Ref	GPCR	Exp. System	Exp. Type	Isotope	Approx. no. assigned peaks	Deuterated protein?	Membrane mimic	Field
Ala	(Kofuku et al., 2018)	$\beta 2\text{AR}$	Sf9	2D	$^1\text{H}/^{13}\text{C}$	8	Yes	Nanodisc (POPC/POPG)	800 MHz
All/backbone	(Tian et al., 2005)	V2R	<i>E. coli</i>	2D	$^1\text{H}/^{15}\text{N}$	0	Yes	Detergent (LMPC)	600 MHz
	(Park et al., 2011)	CXCR1	<i>E. coli</i>	2D	$^1\text{H}/^{15}\text{N}$	14	No	Bicelle (DMPC/POPC)	800 MHz
	(Eddy, Lee, et al., 2018)	A2AR	<i>Pichia</i>	2D	$^1\text{H}/^{15}\text{N}$	14	Yes	Detergent (LMNG/CHS)	800 MHz
	(Eddy, Gao, et al., 2018)	A2AR	<i>Pichia</i>	2D	$^1\text{H}/^{15}\text{N}$	5	Yes	Detergent (LMNG/CHS or DDM/CHS)	800 MHz
	(Franke et al., 2018)	CCR5	Sf9	2D	$^1\text{H}/^{15}\text{N}$	0	Yes	Detergent (DDM)	600 MHz
	(Shiraishi et al., 2018)	$\beta 2\text{AR}$	Sf9, <i>E. coli</i> hybrid	2D	$^1\text{H}/^{13}\text{C}$, $^1\text{H}/^{15}\text{N}$	5 (^{13}C), 22 (^{15}N)	Yes	Nanodisc (POPC/POPG)	800 MHz
Cys	(Klein-Seetharaman, Getmanova, Loewen, Reeves, & Khorana, 1999)	Rhodopsin	Bovine retina	1D	^{19}F	6	No	Detergent (DM, OG, OG/DPMC)	500 MHz
	(Loewen et al., 2001)	Rhodopsin	Bovine retina	1D	^{19}F	2	No	Detergent (DM)	500 MHz
	(Chung et al., 2011)	$\beta 2\text{AR}$	Sf9	1D	^{19}F	1	No	Detergent (DDM or LMNG)	600 MHz
	(J. J. Liu et al., 2012)	$\beta 2\text{AR}$	Sf9	1D	^{19}F	3	No	Detergent (DDM/CHS)	600 MHz
	(Horst, Liu, Stevens, & Wuthrich, 2013)	$\beta 2\text{AR}$	Sf9	1D	^{19}F	2	No	Detergent (DDM/CHS)	600 MHz
	(Kim et al., 2013)	$\beta 2\text{AR}$	Sf9	1D	^{19}F	1	No	Detergent (LMNG)	600 / 500 MHz

Labeled Residue	Ref	GPCR	Exp. System	Exp. Type	Isotope	Approx. no. assigned peaks	Deuterated protein?	Membrane mimic	Field
	(Manglik et al., 2015)	β 2AR	Sf9	1D	^{19}F	1	No	Detergent (LMNG)	600 MHz
	(Eddy et al., 2016)	A2AR	Sf9	1D	^{19}F	2	No	Detergent (DDM/CHS)	600 MHz
	(Staus et al., 2016)	β 2AR	Sf9	1D	^{19}F	1	No	Detergent (LMNG)	600 MHz
	(Ye et al., 2016)	A2AR	<i>Pichia</i>	1D	^{19}F	1	No	Detergent (LMNG/CHS)	600 MHz
	(Ye et al., 2018)	A2AR	<i>Pichia</i>	1D	^{19}F	1	No	Detergent (LMNG/CHS)	600 MHz
Gly	(Klein-Seetharaman et al., 2002)	Rhodopsin	HEK-293	2D	$^1\text{H}/^{13}\text{C}$ (Gly), $^1\text{H}/^{15}\text{N}$ (Lys)	1	No	Detergent (DM)	750 MHz
	(Park et al., 2011)	CXCR1	<i>E. coli</i>	2D	$^1\text{H}/^{15}\text{N}$	14	No	Bicelle (DMPC/POPC)	800 MHz
Ile	(Clark et al., 2017)	A2AR	<i>Pichia</i>	2D	$^1\text{H}/^{13}\text{C}$	4	Yes	Detergent (DDM)	800 MHz
Lys	(Klein-Seetharaman et al., 2002)	Rhodopsin	HEK-293	2D	$^1\text{H}/^{13}\text{C}$ (Gly), $^1\text{H}/^{15}\text{N}$ (Lys)	1	No	Detergent (DM)	750 MHz
	(Bokoch et al., 2010)	β 2AR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	1	No	Detergent (DDM)	800 MHz
	(Sounier et al., 2015)	μ OR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	10	No	Detergent (LMNG/CHS)	800 MHz
Met	(Park et al., 2011)	CXCR1	<i>E. coli</i>	2D	$^1\text{H}/^{15}\text{N}$	14	No	Bicelle (DMPC/POPC)	800 MHz
	(Kofuku et al., 2012)	β 2AR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	9	No	Detergent (DDM)	800 MHz
	(Nygaard et al., 2013)	β 2AR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	4	No	Detergent (DDM/CHS)	800 or 900MHz
	(Kofuku et al., 2014)	β 2AR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	5	Yes	Nanodisc (POPC/POPG)	800 MHz
	(Okude et al., 2015)	μ OR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	6	Yes	Nanodisc (POPC/POPG)	800 MHz
	(Casiraghi et al., 2016)	BLT2	<i>E. coli</i>	2D	$^1\text{H}/^{13}\text{C}$	2	Yes	Nanodisc (DMPC/CHS)	800 MHz
	(Solt et al., 2017)	β 2AR	Sf9	2D	$^1\text{H}/^{13}\text{C}$	8	No	Detergent (LMNG)	800 MHz
	(Bumbak et al., 2018)	NTS1	<i>E. coli</i>	2D	$^1\text{H}/^{13}\text{C}$	0	No	Detergent (DDM)	800 MHz
	(Korczynska et al., 2018)	M2R	Sf9	2D	$^1\text{H}/^{13}\text{C}$	5	No	Detergent (LMNG/CHS)	800 MHz

Labeled Residue	Ref	GPCR	Exp. System	Exp. Type	Isotope	Approx. no. assigned peaks	Deuterated protein?	Membrane mimic	Field
	(Shiraishi et al., 2018)	β 2AR	Sf9, <i>E. coli</i> hybrid	2D	$^1\text{H}/^{13}\text{C}$, $^1\text{H}/^{15}\text{N}$	5 (^{13}C), 22 (^{15}N)	Yes	Nanodisc (POPC/POPG)	800 MHz
Trp	(Klein-Seetharaman et al., 2004)	Rhodopsin	HEK-293	2D	$^1\text{H}/^{15}\text{N}$	0	No	Detergent (DM)	800 MHz
	(Stehle et al., 2014)	Meta-rhodopsin	HEK-293	2D	$^1\text{H}/^{15}\text{N}$	5	No	Detergent (DDM)	800 MHz
	(Eddy, Gao, et al., 2018)	A2AR	<i>Pichia</i>	2D	$^1\text{H}/^{15}\text{N}$	5	Yes	Detergent (LMNG/CHS or DDM/CHS)	800 MHz
Val	(Park et al., 2011)	CXCR1	<i>E. coli</i>	2D	$^1\text{H}/^{15}\text{N}$	14	No	Bicelle (DMPC/POPC)	800 MHz
	(Isogai et al., 2016)	β 1AR	Sf9	2D	$^1\text{H}/^{15}\text{N}$	21	No	Detergent (DM)	900 MHz