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Characterization and assignment of uniformly labeled NT(8-13) at the agonist binding site of the G-protein coupled neurotensin receptor

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Introduction

The neurotensin receptor is a member of the G-protein coupled receptor (GPCR) family of transmembrane proteins that is activated upon the binding of the basic tridecapeptide agonist, neurotensin, to the extracellular surfaces of cells. The neurotensin receptor is found widely in both the central nervous system and the periphery. In the periphery, it stimulates smooth muscle contraction [1,2]. In the central nervous system, it mediates a variety of activities including antinociception, hypothermia and increased locomoter activity [3-5]. These effects are probably mediated through the regulation of the mesolimbic and negrostriatal dopamine pathways [6,7]. As a result, the pharmacological action of the neurotensin is similar to that observed for dopamine, where compounds function as antipsychotics [6,7], and intervention may provide useful insights for the development of treatments for conditions such as schizophrenia [8] and Parkinson's disease [9].

To date no direct high resolution structural information is available for the neurotensin receptor due to limited successes at production of 2D and 3D crystals for diffraction studies and to the unfavorable relaxation rates associated with this size of membrane system in conventional high resolution solution state NMR studies. Although no direct structural information is available for the neurotensin receptors, holistic modeling approaches have been employed to provide evidence that the receptor adopts the typical 7 transmembrane motif shared by this family of receptors [10]. A putative agonist binding site containing the agonist analogue neurotensin(8-13) has been modeled, supported by site directed mutagenesis and structure/activity studies [11]. High-resolution solution NMR structural studies of the agonist, neurotensin, in the absence of receptor have revealed that no preferred conformation exists in solution [12]. Extensions of these studies to neurotensin in the presence of the membrane mimetic sodium dodecyl d_{25}-sulphate again indicate that no preferred conformation was adopted although some ordering of charged residues on the surface of the micelles was observed [12].

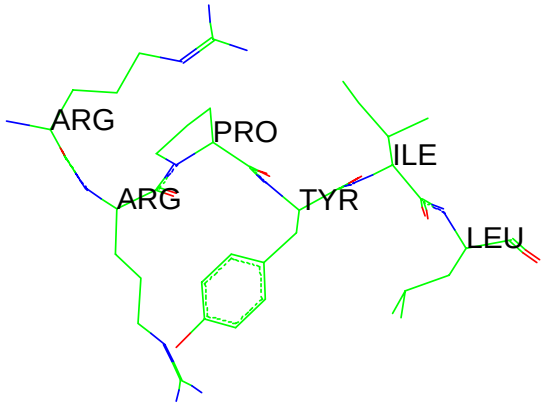

Fig 1. Diagram showing an energy minimized structure of neurotensin(8-13) (Arg[1]-Arg[2]-Pro[3]-Tyr[4]-Ile[5]-Leu[6]), a C-terminal fragment of neurotensin that shows similar pharmacological efficacy (K_d=13nM).

The aim of the studies presented here is to provide direct structural information for the agonist, neurotensin, whilst bound to the functionally active neurotensin receptor. This information may be included in future models to aid the understanding of the events associated with the molecular recognition of neurotensin by the neurotensin receptor. To this end we have undertaken the preparation of sufficient quantities of recombinant neurotensin receptor to support a solid state NMR study. Through the incorporation of both carbon-13 and nitrogen-15 into a pharmacologically active C-terminal fragment of neurotensin, neurotensin(8-13) (Fig 1), we have been able to employ solid state NMR methodologies to specifically observe neurotensin(8-13) whilst bound to the neurotensin receptor and to assign resonances to particular groups within the agonist. As a prelude to a full assignment of neurotensin(8-13) bound to the receptor, we have performed a near complete assignment of the lyophilized agonist fragment. This demonstrates that sufficient resolution exists to permit a full assignment of such compounds. These assignments are essential to further structural work and may, under favorable conditions, allow us to suggest a preferred conformation of the agonist whilst bound to the receptor.

Materials and Methods

Expression of neurotensin receptor

Detergent solubilized rat neurotensin receptor was obtained by the method of Grisshammer [13]. For expression *E.coli* strain DH5α was grown on double strength TY medium [14] containing ampicillin (100 μg/ml) and 0.2% glucose. A NTR fusion protein was expressed from the pRG/III-hs-MBPP-T43NTR-TrxA-H10 gene construct kindly donated by Dr R. Grisshammer [13]. This plasmid contains a truncated neurotensin receptor, which is fused at its N-terminus to a maltose binding protein and its signal sequence (MBP) in order to target the N terminus to the periplasm. It is also

fused at its C-terminus to thioredoxin, to aid stability, and to a deca-his tag to assist in purification. The transformed DH5α were grown in 400ml of medium in a 1 l flask at 37°C until OD660 reached 0.7. The cultures were subsequently induced with 0.5mM isopropyl-β-galactoside (IPTG). The temperature was then lowered to 20°C and incubated for a further 40 h. The cells were harvested by centrifugation, flash frozen in liquid nitrogen and stored at –70°C.

Purification of neurotensin receptor

The purification of heterologously expressed neurotensin receptor was performed using the method of Grisshammer [15]. Briefly, 200g of cell paste were resuspended in 1.2l of neurotensin buffer (50mM TRIS, 0.2M NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% LM) containing pepstatin A, leupeptin A, PMSF, lysozyme and Dnase, to prevent protein degradation and to aid cell lysis. The cells were then broken by three passes through a flow-through sonicator and subsequently clarified by centrifugation. The supernatent was loaded onto a Quiagen NTA nickel affinity column in 1mM imidazole at a flow rate of 10 ml min^{-1}. The neurotensin receptor eluted with neurotensin buffer containing 350 mM imidazole. The eluate was then concentrated using an Amicon stirred cell with YM-30 membrane.

The buffer was subsequently exchanged to a low salt buffer (50mM TRIS, 20mM NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% LM) using a 150 ml Sephadex-G25 column. The active receptor was purified using a neurotensin affinity column[13]. The affinity column was equilibrated with low salt buffer and the fraction containing the neurotensin receptor loaded at 0.5 ml min^{-1}. Following washing with both low salt buffer and 200 mM KCl buffer, the active neurotensin receptor was eluted using a high salt buffer (50 mM TRIS, 1.0 M NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% LM). Prior to NMR studies the purified neurotensin receptor was returned to desalting buffer and concentrated using a stirred cell Amicon and Centricon containing a YM-30 membrane.

The activity of the protein was monitored using a tritiated-neurotensin binding assay [15], whilst the protein concentration was monitored using an amido black protein assay [15]. Purity was determined by a 5-12% gradient SDS-PAGE, followed by Comassie staining [15].

Solid phase synthesis of neurotensin(8-13).

Neurotensin(8-13) was synthesized using conventional FMOC solid phase synthesis at the Oxford Center for Molecular Sciences. Uniformly 13C and 15N labeled amino acids (Promochem, UK) were protected and purified using standard amino acid protection protocols [16,17].The compounds confirmed by electrospray mass spectroscopy and thin layer chromatography [17]. Following solid phase synthesis, the peptide was purified by reverse phase HPLC, eluting at an acetonitrile concentration of

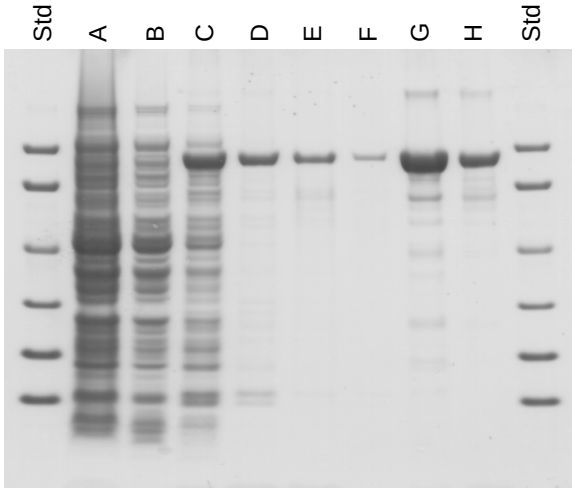

Fig 2. 4-12% Comassie stained MES-SDS polyacrylamide gradient gel of samples obtained during the purification of recombinant neurotensin receptor. lanes: standards (Std), clarified sample(A), Quiagen-NTA flow through (B), 500mM imidazole eluate (C), desalted sample (D), NT column flow through (E), 200mM KCl wash (F), 1M NaCl eluate from NT affinity column (G) and concentrated sample of neurotensin receptor (H).

27% comparable with unlabeled neurotensin(8-13) (Sigma, UK) used as a standard. Electrospray mass spectroscopy of the final product gave a single molecular species with molecular weight 868 Da consistent with uniform 13C and 15N isotopic labeling of neurotensin(8-13).

NMR methods

CP-MAS spectra of the lyophilized samples of neurotensin(8-13) were acquired on a Bruker Avance 600, operating at 600 MHz (proton Lamour frequency) with a 2.5mm Bruker triple resonance probehead. Carbon-13 and Nitrogen-15 cross polarization magic angle spinning (CP-MAS) spectra were acquired using an adiabatic cross polarization sequence [18] with a constant proton field of 60 kHz. Decoupling during acquisition was performed with 125 kHz TPPM decoupling [19] (phase alternation 7°). Carbon-13/Nitrogen-15 HETCOR experiments was performed at 15 kHz spinning, and transfer from Nitrogen-15 and Carbon-13 was achieved by an adiabatic sweep (10ms, centered at 50 kHz field). During t_1 and t_2, TPPM decoupling was applied as described above, and Lee Goldberg decoupling with an applied field of 120kHz was employed during mixing. Phase sensitive detection in t_1 was achieved using TPPI phase cycling of the initial proton to nitrogen-15 cross polarization. Carbon-13/Carbon-13 correlation data was acquired using a 2D exchange experiment at 22.5 kHz spinning with a RFDR sequence [20] (π pulses of 8μs were applied rotor synchronously with XY-8 phase cycling) applied during the mixing period. During t_1 and t_2, TPPM decoupling was applied as described, and 120 kHz Lee-Goldberg decoupling was applied during mixing.

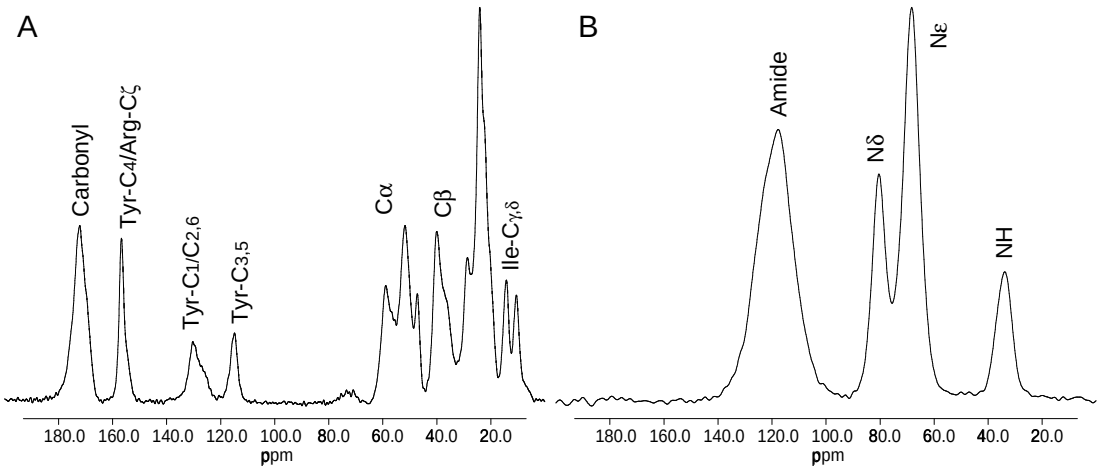

Fig 3. Carbon-13 (A) and nitrogen-15 (B) CP-MAS spectra of neurotensin(8-13) uniformly labeled with carbon-13 and nitrogen-15. Both spectra acquired with CP-MAS at 15 kHz spinning speed with other parameters as described in the text. Data accumulated over 128 and 256 acquisition respectively and processed with 30 Hz linebroadening.

Carbon-13 CP-MAS spectra of detergent solubilized receptor were obtained on a Chemagnetics CMX-500 operating at 500 MHz (proton Lamour frequency), with a Chemagnetics 6mm triple resonance probehead. CP-MAS spectra were acquired using an adiabatic cross polarization sequence with a proton field of 60 kHz. During acquisition protons were decoupled using 80 kHz continuous wave irradiation. Double quantum-filtered experiments were performed using the POST-C7 sequence [21]. The carbon-13 B_1 field (35kHz) was matched to seven times the rotor speed and 10 C7 elements were applied for both excitation and reconversion. Double quantum coherence is selectively observed through the appropriate phase cycling of the C7 reconversion sequence, the final $\pi/2$ pulse and the receiver [21]. During the C7 sequence the protons were decoupled using Lee-Goldberg decoupling with an applied field of 80 kHz.

Results & Discussion

Purification of detergent solubilized neurotensin receptor.

Recombinant neurotensin receptor was purified from 230 l of culture (~1.4kg wet cell paste) in seven batches. Typically crude cell lysate contained neurotensin receptor at 1-3 pmoles mg^{-1}. A 200 fold enrichment in the neurotensin receptor (~600 pmoles mg^{-1}) was achieved by Ni affinity purification, which removed the bulk of the contaminating proteins. The remaining contaminants were non-function neurotensin receptor and endogenous *E.coli* Ni affinity binding proteins. These were removed by purification on a neurotensin affinity column, which resulted in a further 3.5 fold enrichment of the neurotensin receptor (specific activity 2500 pmoles mg^{-1}). The course of the purification and final purity of the recombinant neurotensin receptor was monitored by SDS-PAGE and is shown in Figure 2 (Final sample, Lane H).

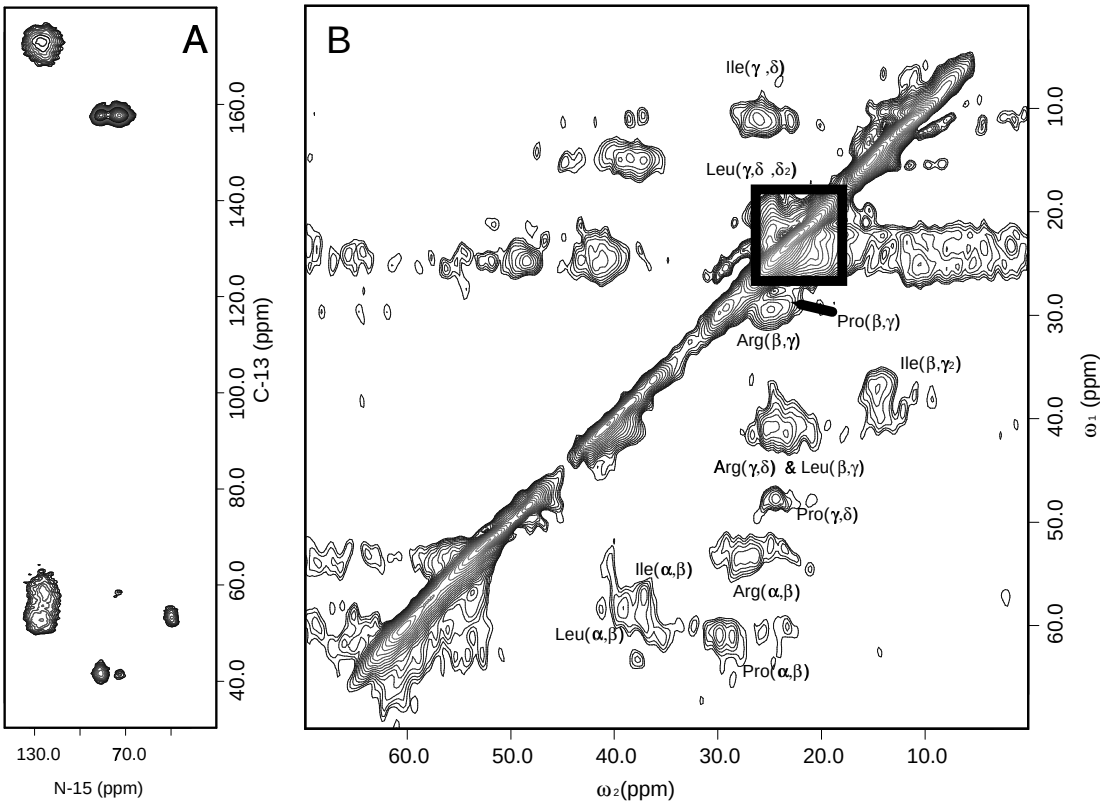

Fig 4. Nitrogen-15/Carbon-13 heteronuclear correlation experiment of lyophilized neurotensin(8-13)(A). Data acquired with 128 t_1 points with 64 acquisitions for each point. Data processed with 50 Hz linebroadening prior to Fourier transform. Data in t_1 was then linear predicted from 128 to 512 points and a sinebell function applied prior to a real Fourier transform in t_1. Final matrix 2048x2048 points. Expansion of the sidechain region of a 13C-13C-correlation spectrum (B) of lyophilized neurotensin(8-13) with 2.3 ms exchange at 22.5 kHz. Data acquired with 256 t_1 points and 128 acquisitions of each. Data processed with 30 Hz linebroadening in t_2. Data was linear predicted from 256 to 1024 points and processed with a sinebell function prior to Fourier transformation in t_1. Final matrix 2048x2048 points.

Assignment of neurotensin(8-13).

The carbon-13 and nitrogen-15 CP-MAS spectra of neurotensin(8-13) are shown in Figures 3A and 3B, respectively. Although in the 1D CP-MAS spectra individual sites are rarely resolved due to the large linewidths observed in this heterogeneous sample of lyophilized neurotensin(8-13), these broad resonances can be assigned to the individual functional groups which contribute to the neurotensin(8-13) (Fig 3A and 3B). In the carbon spectrum, several sites can be assigned on the basis of their unique chemical shifts including the tyrosine, arginine and some of the aliphatic side chains. However many of the resonances associated with the peptide backbone are poorly resolved due to the relatively small chemical shift dispersion and the relatively large linewidths

associated with the sample. A similar situation is apparent in the nitrogen-15 spectrum where the arginine sidechains and terminal amine are clearly visible while poor resolution is apparent in the amide region centered at 125 ppm. To circumvent these problems of poor resolution and to provide essential correlation data to allow the assignment of these 1D spectra, both homo- and heteronuclear correlation spectra were acquired.

The nitrogen-15/carbon-13 correlation spectrum is shown in Figure 4A. The spectrum is dominated by the strong correlations between the amide nitrogen (120ppm) and the carbonyl (175ppm) and C_α resonances (~52ppm). In these regions, areas of heightened intensity can be seen that correspond to the C_α/CO correlations observed in the homonuclear correlation spectra (See below). In addition strong correlations are also observed between the N-terminal amine (~37ppm) and the C_α of Arg_1 (53.3ppm). Strong correlations between the two inequivalent nitrogens of the arginine sidechain, N_ε (~84ppm) and N_δ (~70ppm), and the C_ζ carbon are also apparent. The N_δ can then be traced to C_δ(40.9ppm). Under the long mixing conditions employed here, it is also apparent that long range transfer occurs between the N_ε and the C_δ. Whether this is due to relayed transfer along the chain or to direct through space transfer is unclear.

An expansion of the sidechain region (0-70ppm) of a carbon-13/carbon-13 correlation spectrum of neurotensin(8-13) is shown in Figure 4B. Under the regime chosen with short exchange times, the intensities observed arise almost solely from correlation's between direct carbon-carbon pairs. Several regions of intensity in the spectrum arise primarily from the C_α/CO connectivities: the tyrosine ring region and the upfield region assigned to the C_α and aliphatic sidechains. On the basis of the unique chemical shifts arising from several of the sites in the sidechains, it has been possible to assign most of the sidechain resonances and to correlate them to the backbone C_α and CO resonances,

Table 1. Carbon-13 assignments for lyophilized neurotensin(8-13) obtained from both homo- and heteronuclear correlation experiments.

	Carbon-13 chemical shift (±1.0ppm)							
Residue	CO	C_α	C_β	C_γ	C_δ	C_ε	C_ξ	Other
Arg_1	169.6	53.3	28.9	24.5	40.9		157.6	
Arg_2		52.6	28.9	24.5	40.9		157.6	
Pro_3	172.3	61.1	29.5	24.1	48.0			
Tyr_4	174.2	52.8	N/A					127.9 (C1) 130.7(C2,6) 115.7(C3,5) 155.6(C4)
Ile_5	172.3	57.4	36.8	25.6 14.2	11.0			
Leu_6	172.5	53.4	38.7	23.7	18-27 18-27			

allowing an almost complete assignment of the lyophilized neurotensin(8-13). The results of this assignment are shown in Table 1. With the exception of Tyr_{β} and the Leu_{δ},it has been possible to assign all sites unambiguously. We attribute our inability to assign the two Leu C_{δ} to the relatively small chemical shift dispersion expected between the two resonances which prohibits their resolution against the more intense diagonal elements in the spectrum. Hightened intensity in this region does indicate that these sites are correlated (Figure 4A, box). In contrast the absence of the Tyr-C_{β} assignment arises due to the absence of any intenisty in the Tyr-C_{β}/Tyr-C1 region possibly due to unfavourable relaxation under the mixing sequence chosen.

Assignment of neurotensin(8-13) bound to detergent solubilized neurotensin receptor.

The carbon-13 CP-MAS spectrum of detergent solubilized neurotensin receptor is shown in Fig 5A. The spectrum is dominated by the strong natural abundance signals arising from the detergents (170-180ppm, ~100ppm and 10-50ppm) and glycerol (62 and 71 ppm) present in the sample. Upon the addition of a stoichiometric amount of neurotensin(8-13) (10 nmoles, <5% free ligand), the major spectral features remain constant (Fig 5B). In regions displaying greater spectral complexity (0-70 ppm), we are unable to identify any additional resonances which may arise from the C_{α}, C_{β}, and C_{γ} resonances of the ligand as they are masked by the natural abundance signal from the buffer components. In regions that are less crowded, such as between 169 and 175 ppm and 152 and 157 ppm, spectral perturbations are observed. The spectral intensity arising between 169 and 175ppm has been assigned to the labeled carbonyl groups present in the peptide, whilst the spectral intensity between 152 and 157ppm has been assigned to the C_{ξ} of the arginine sidechains and the C4 of the tyrosine sidechains on the basis of the observed chemical shifts. CP-MAS studies of NT(8-13) in identical buffers at these temperatures revealed no such intensities suggesting that the signal arising in these spectra occur due to the additional immobilization inferred to the ligand upon binding to the detergent solubilized receptor. Additionally higher resolution spectra acquired with direct carbon excitation and MAS at 5°C indicated significant perturbations in chemical shift upon the binding of the neurotensin(8-13) to the receptor [17] (data not shown). From these studies we conclude that the resonances observed arise solely from the neurotensin(8-13) specifically bound to the neurotensin receptor

To resolve sites masked by the background natural abundance signal from the detergents, double quantum filtered spectra of the sample were obtained (Fig 5C) through the reintroduction of dipolar coupling using the POST-C7 sequence. Through the specific observation of only those nuclei that have strong carbon-carbon couplings, it has been possible to suppress the large natural abundance background signal sufficiently (as only 0.01% of natural abundance carbon atoms share labeled neighbors). This has allowed us to observe resonances arising primarily from the C_{α} (50-60ppm) and from sites in the aliphatic sidechains (10-50ppm) within the peptide in addition to those previously observed for both the aromatic and basic sidechains. The linewidths

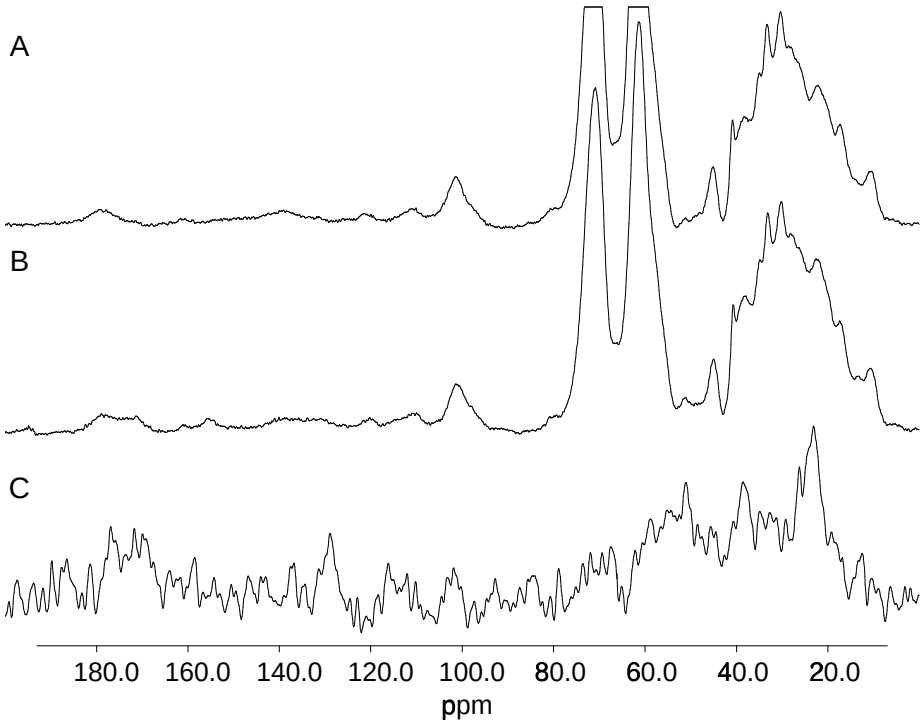

Fig 5. Carbon-13 CP-MAS spectra of 1 mg of detergent solubilized neurotensin receptor (A) and upon the addition of a stoichiometric amount of neurotensin(8-13) (10 nmoles) (B). Data acquired with a 1ms contact time, 5000 Hz spinning speed and 70 kHz decoupling. POST-C7 double quantum filtered spectra of 1 mg of detergent solubilized neurotensin receptor containing 10 nmoles of uniformly labeled neurotensin(8-13), acquired with a 512 μs excitation and reconversion period. Data averaged over 8192 acquisitions and processed with 30 Hz linebroadening (C - 100Hz linebroadening).

observed for the bound neurotensin(8-13) in the 1D CP-MAS experiments are similar to those observed for neurotensin(8-13). As we have demonstrated for the lyophilized sample above, these conditions are sufficient to allow a near complete assignment of the neurotensin(8-13) whilst bound to the neurotensin receptor under solid state conditions.

Conclusions.

The data presented here demonstrates that we are able to suppress large natural abundance background signals to selectively observe the agonist analogue neurotensin(8-13) whilst resident in the agonist binding site on the detergent solubilized neurotensin receptor. This is the first time an exchangeable ligand has been observed whilst bound to a GPCR by solid state NMR. Even in the absence of a suitably crystalline sample, through the application of homo-nuclear and hetero-nuclear correlation spectroscopy, we have demonstrated that the methodology and the resolution exists to permit a full assignment of the neurotensin(8-13) whilst bound to the receptor under solid state conditions. This data demonstrates that upon the successful

reconstitution of the the neurotensin receptor into lipid bilayers the methodology exists to permit a partial assignment of the bound neurotensin(8-13) whilst resident in the agonist binding site of the reconstituted receptor. Prior to further structural studies, we aim to exploit the chemical shifts observed to provide information relating to the conformation of the ligand as a lyophilized powder and whilst bound to the receptor.

Acknowledgements.

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