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Identification and importance of *N*-glycosylation of the human 5-hydroxytryptamine$_{3A}$ receptor subunit

Sarah A. Monk, Julie M. Williams, Anthony G. Hope[1], Nicholas M. Barnes*

Cellular and Molecular Neuropharmacology Research Group, Department of Pharmacology, Division of Neuroscience, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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Abstract

We investigated the presence and potential role of *N*-glycosylation of the human (h) 5-hydroxytryptamine$_3$ (5-HT$_{3A}$) receptor subunit expressed in COS-7 cells. Incubation of cells with the *N*-glycosylation inhibitor, tunicamycin, reduced the molecular weight of the predominant immunoreactive h5-HT$_{3A}$ subunit species (from ≈59 to 45 kDa) indicating that the h5-HT$_{3A}$ subunit is normally *N*-glycosylated. Site-directed mutagenesis studies individually substituting four identified N-terminal asparagines (N5, N81, N147, N163) demonstrated that each expressed mutant displayed a reduced molecular weight (by ≈3 kDa) suggesting that each asparagine residue was subject to *N*-glycosylation. In addition, 5-HT$_3$ receptor binding studies indicated that prevention of *N*-glycosylation, by individual amino acid substitution at each of the four asparagine residues, either prevented (N81, N147, N163) or greatly reduced (N5) the production of a 5-HT$_3$ receptor binding site. Corresponding with the radioligand binding studies, immunocytochemical studies demonstrated that substitution of each asparagine either prevented (N81, N147, N163) or reduced considerably (N5) mutant protein expression within the cell membrane. The present study demonstrates an important role for *N*-glycosylation at multiple identified asparagine residues in the N-terminus of the h5-HT$_{3A}$ receptor subunit.

Keywords: 5-HT$_3$ receptor; 5-HT$_{3A}$ subunit; Glycosylation; Post-translational modification; Ligand-gated ion channel; 5-Hydroxytryptamine

1. Introduction

The 5-HT$_3$ receptor is a LGIC that mediates fast synaptic neurotransmission in central and peripheral neurones (for review see [1]). In common with other members of the cys–cys loop LGICs (e.g. nicotinic acetylcholine receptor, GABA$_A$ receptor, ionotropic glycine receptor), functional receptors are likely to be pentameric protein complexes forming a central ion channel, which in the case of the 5-HT$_3$ receptor, primarily conducts cations (e.g. Na^+, K^+, Ca^{2+}) resulting in neuronal depolarisation upon activation.

Whilst the expression of the first identified 5-HT$_3$ protein subunit, the 5-HT$_{3A}$ subunit, generates a functional receptor complex mimicking many of the characteristics of some native 5-HT$_3$ receptors, certain native receptor populations differ from these homomeric 5-HT$_{3A}$ receptor complexes and are likely to additionally incorporate further proteins such as the 5-HT$_{3B}$ receptor subunit [2,3]. This latter subunit fails to form functional homomeric 5-HT$_{3B}$ receptors, nor indeed a ligand binding site, but when co-expressed with the 5-HT$_{3A}$ receptor subunit, the resulting presumably heteromeric 5-HT$_{3A/3B}$ receptor complex, displays higher single channel conductance consistent with some populations of native 5-HT$_3$ receptors [2]. The potential for molecular diversity has recently been further extended with the identification of three genes that may generate additional 5-HT$_3$ receptor subunits (*5-HT$_{3C}$*, *5-HT$_{3D}$* and *5-HT$_{3E}$*; [4,5]), although their potential contribution to 5-HT$_3$ receptor diversity remains to be elucidated.

In addition to the formation of 5-HT$_3$ receptor isoforms with differing subunit compositions, differential post-translational modifications may also contribute to receptor diversity. The clear precedence for other members of the

Abbreviations: h, Human; 5-HT, 5-hydroxytryptamine; LGIC, ligand-gated ion channel; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline-Tween; TX100, Triton X-100.

* Corresponding author. Tel.: +44 121 414 4499; fax: +44 121 414 4509.
E-mail address: n.m.barnes@bham.ac.uk (N.M. Barnes).
[1] Present address: Organon Laboratories Ltd., Newhouse, Lanarkshire, Scotland ML1 5SH.

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cys–cys loop LGIC to be differentially modified by phosphorylation (e.g. [6,7]) and glycosylation (e.g. [8,9]) suggests that these processes will also be important to 5-HT_3 receptor function. Indeed, phosphorylation of the 5-HT_3 receptor protein, or associating proteins, has already been demonstrated by a number of independent laboratories (e.g. [10–14]).

In the present report, we document the ability of the h5-HT_{3A} receptor subunit to be *N*-glycosylated at four identified amino acid residues in the extracellular N-terminus and demonstrate that failure to glycosylate each target either reduces or prevents membrane expression of the h5-HT_{3A} subunit and the formation of a 5-HT_3 receptor binding site.

2. Methods

2.1. Construction of an epitope-tagged human 5-HT_{3A} subunit

The human 5-HT_{3A} subunit cDNA was cloned into the vector pcDNA3.1 (Invitrogen) and tagged with the human c-myc epitope (EQKLISEEDL) by insertion of the encoding DNA (GAA-CAA-AAA-CTC-ATC-TCA-GAA-GAG-GAT-CTG) between the fourth and fifth amino acids of the mature protein sequence (Fig. 1).

2.2. Transient transfection of COS-7 cells

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and maintained at 37 °C, 5% CO_2, 95% relative humidity.

COS-7 cells (5×10^6) were transiently transfected with 30 μg cDNA via electroporation (400 V, 150 μF, in 4 mm path length cuvettes). Transfected cells were seeded at a density of 2.4×10^6 cells/100 mm cell culture dishes (for SDS-PAGE/western blotting and radioligand binding) or at a density of 2.4×10^4 cells/ml in 8-well chamber glass slides (500 μl per well) for immunocytochemical studies. For SDS-PAGE/western blotting and radioligand binding studies, cells were harvested 48 h post-transfection by scraping cells into PBS and cell pellets recovered by centrifugation at 3700 × *g* for 5 min at room temperature. For immunocytochemical studies, cells were fixed (formaldehyde (4%) in PBS; 20 min, room temperature) 48 h post-seeding. The cells were stored at −80 °C prior to use (typically less than 1 week).

2.3. SDS-PAGE and western blotting

COS-7 cell homogenates were prepared by resuspension of the cell pellets in ice-cold Tris–PI buffer (50 mM, pH 7.4; containing various protease inhibitors (at final concentration of 10 μg/ml for aprotinin, leupeptin and pepstatin, and 2 mM phenylmethylsulfonylfluoride (PMSF)) before sonication (Kerry Ultrasonics; full power for 10 s over ice) followed by centrifugation (18,000 × *g*; 10 min at 4 °C). The pellets were washed a further three times by resuspension-centrifugation before final resuspension in ice-cold Tris buffer (50 mM; pH 7.4). Protein concentrations were determined using Coomassie Blue dye (Bio-Rad) using bovine serum albumin as the standard.

Cell homogenate samples were subject to separation by SDS-PAGE under reducing conditions followed by western blotting as described previously [15]. Briefly, homogenate aliquots of equal protein concentrations were combined with an equal volume of 2× sample buffer (125 mM Tris pH 6.8, 20% (w/v) glycerol, 4% SDS, 10% (3-mercaptoethanol, 0.02% bromophenol blue). Samples (15 μl) were loaded onto a 10% SDS PAGE gel (run at 20 mA per gel) to separate proteins. Following protein transfer to PVDF membrane, the membrane was blocked at room temperature (3 h) with bovine serum albumin (5% (w/v) in TBS-T (Tris 20 mM, NaCl 140 mM, Tween 0.1% (v/v)) to reduce non-specific antibody binding to the membrane. The myc-epitope was immunologically labelled by incubation with monoclonal anti-myc antibody (clone 9B11, cell signalling; 1:1000 dilution in blocking buffer for 16 h at 4 °C), followed by extensive washing with TBS-T prior to incubation (1 h, room temperature) with the secondary antibody (goat anti-mouse IgG HRP-linked, cell signalling; 1:2000 dilution in TBS-T containing dried milk powder 5% (w/v)). The blots were further washed in TBS-T and subsequently incubated in Enhanced Chemiluminescent Solution (Supersignal® West Pico Chemiluminescent Substrate, Pierce) to allow visualisation of immunoreactive proteins using Hyperfilm ECL (Amersham).

2.4. Radioligand binding

Radioligand binding assays were performed as previously described in detail [16]. Briefly, tubes (in at least duplicate) contained 100 μl of competing drug (for competition studies, drugs were used at a range of concentrations or for detection of specific 5-HT_3 receptor binding, ondansetron, at a final concentration of 10 μM to define non-specific binding which was less than 10% of the total binding in all of the experiments reported in the present paper) or vehicle (50 mM Tris, pH 7.4; total binding) and 100 μl of [^{3}H]granisetron (generally ≈1–5 nM but for some experiments ≈11 nM; NEN, 82 Ci mmol^{-1}). An aliquot (100 μl) of the appropriate membrane fraction was added to initiate binding, which was allowed to proceed at room temperature for 60–90 min before termination by rapid filtration and washing under vacuum through Whatman GF/B filters, followed by assay of the radioactivity remaining on the filters.

Fig. 1. Amino acid sequence of the mature extracellular N-terminus of the h5-HT_{3A} receptor subunit protein highlighting the four potential *N*-glycosylation sites (indicated *; consensus *N*-glycosylation motif, N-X-S/T, underlined with the asparagine residue, N, in bold). The location of the inserted myc-epitope tag is also displayed between the fourth and fifth amino acids of the protein. TMD-I, putative transmembrane domain I. Numbering is in accordance with the mature peptide sequence numbering [29] excluding the 10 amino acid myc-epitope tag insertion).

2.5. Immunocytochemistry

Formaldehyde fixed transiently transfected COS-7 cells, adhered to microscope slides, were extensively washed (PBS) at room temperature. Cells were then incubated in PBS ± TX100 (0.3%, NB; the presence of the detergent, TX100, would allow antibody to penetrate the intracellular compartment) for 1 h at room temperature prior to incubation in PBS/normal horse serum (3%) ± TX100 (0.3%) for a further hour at room temperature before incubation in 1° antibody (monoclonal anti-myc clone 9B11, cell signalling; 1:1000 diluted in PBS/normal horse serum (3%) ± TX100 (0.3%)) overnight at 4 °C. The cells were then washed extensively in PBS ± TX100 (0.3%) prior to incubation in secondary antibody (1:200–300 horse anti-mouse fluorescein-labelled; Vector) in PBS/normal horse serum (3%) ± TX100 (0.3%) for 2 h at room temperature. Following extensive washing in PBS ± TX100 (0.3%), cells were incubated in PBS/TX100 (0.3%) for 1 h and then PBS/normal goat serum (3%)/TX100 (0.3%)/bovine serum albumin (BSA; 5%) for a further hour at room temperature before being incubated overnight (4 °C) with the second primary antibody (1:1000 rabbit anti-5-HT_{3A} subunit; a generous gift from McKernan and co-workers [17]) in PBS/normal goat serum (3%)/TX100 (0.3%)/BSA (5%). Following extensive washing in PBS/TX100 (0.3%), cells were incubated with a secondary antibody directed against the anti-5-HT_{3A} antibody (1:200 goat anti-rabbit Alexa-Fluor 568; Molecular Probes) in PBS/normal goat serum (3%)/TX100 (0.3%)/BSA (5%) for 2 h at room temperature. The cells were then mounted following extensive washing and viewed with an epifluorescence microscope. Images were captured using a digital camera with Lucia (Nikon) software.

2.6. Site-directed mutagenesis

Analysis of the extracellular N-terminal amino acid sequence of the h5-HT_{3A} receptor subunit revealed four potential *N*-glycosylation sites (consensus sequence NXS/T, where X is any amino acid except proline [18]); N5, N81, N147 and N163 (Fig. 1).

Site-directed mutagenesis was achieved for each identified position (except for N81) using the QuikChange method (Stratagene) in accordance with the manufacturer's instructions, using the following temperature cycling parameters: one cycle at 95 °C for 30 s followed by 18 cycles at 95 °C for 30 s, 55 °C for 1 min, 68 °C for 14 min. Samples

were then held at 4 °C prior to digestion and transformation into *E. coli* (strain XL 1 blue). N81 was mutated by the method of Kunkel [19], using a single-stranded DNA template (incorporating the coding sequence of h5-HT_{3A}) synthesised from the M13 of pcDNA3.1.

As in previous studies (e.g. [20]), mutagenic oligonucleotides were designed to substitute asparagine (N) with serine (S) residues in order to disrupt the consensus *N*-glycosylation sequence motif, and hence prevent *N*-glycosylation at the appropriate position in the expressed mutated protein. In addition, primers included a silent mutation encoding a restriction site (shown in bold) to facilitate screening of mutant h5-HT_{3A}-myc cDNAs. The forward mutagenic oligonucleotide sequences used for QuikChange mutagenesis are listed below (asparagine-to-serine mutations are underlined). For N81S, the mutagenic oligonucleotide is expressed as non-coding sequence:

N5S 5′-CAT-CTC-AGA-GGA-TCT-**GTC**-**G**AC-CAC-CAG-GCC-CGC-TCT-G-3′ (Sal I)
N81S 5′-CTT-GGT-GAT-GCT-GTC-AAA-GTC-**CTC**-**GGG**-GTT-CCA-CT-3′ (Ava I)
N147S 5′-GAT-GTC-CAG-AGC-TGC-TC**G**-**TTG**-**AC**C-TTC-AC-3′ (Hinc II)
N163S 5′-CAG-GAC-ATC-AGC-ATC-TCT-TTG-T**GG**-**CGC**-**C**TG-CCA-GAA-AA-3′ (Nar I)

Verification of the desired site mutations were determined by commercial fluorescent automated DNA sequencing of the purified DNA preparations (Lark Technologies Inc.).

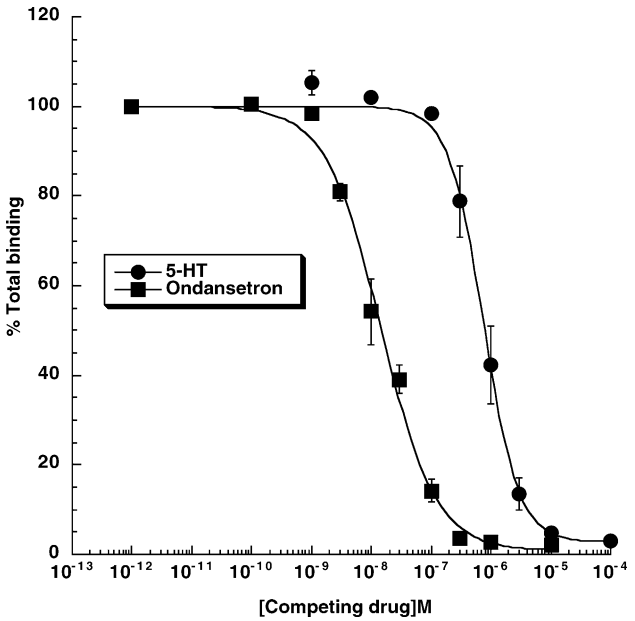

Fig. 2. Ability of the selective 5-HT_3 receptor antagonist, ondansetron, and the natural agonist, 5-HT, to compete for [^{3}H]granisetron (1–2 nM; total binding in individual assays was between 30,000 and 36,000 dpm) binding to homogenates derived from COS-7 cells transiently transfected with the cDNA encoding the myc-tagged h5-HT_{3A} receptor subunit. Data represent mean ± SEM from three experiments.

3. Results

Radioligand binding studies demonstrated that the incorporation of the myc-epitope between the fourth and fifth amino acids of the h5-HT_{3A} subunit (h5-HT_{3A}-myc)

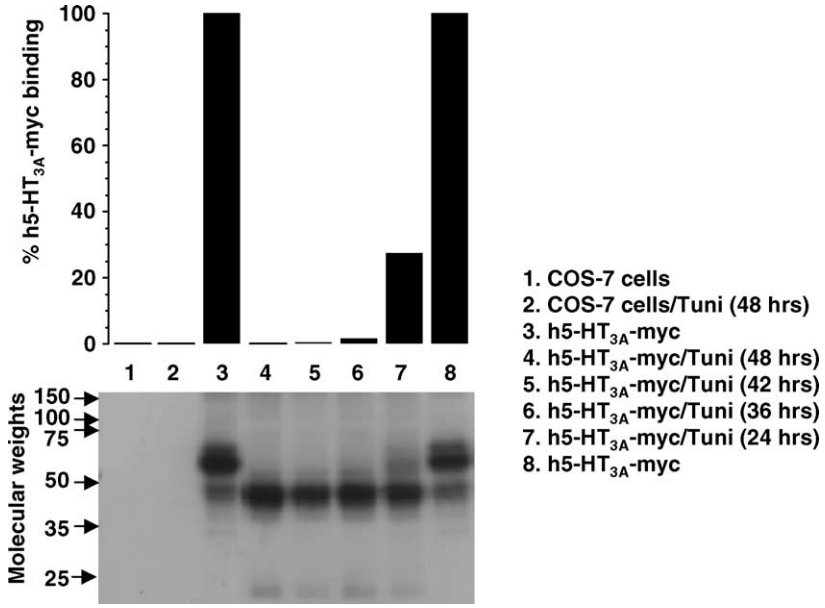

Fig. 3. Effect of time of incubation with the *N*-glycosylation inhibitor, tunicamycin (1.0 μg/ml), on specific [^{3}H]granisetron 5-HT_3 receptor binding (top panel) and apparent molecular weights of myc-immunoreactive protein species (western blot; bottom panel) resulting from COS-7 cells transiently transfected with cDNA encoding the h5-HT_{3A}-myc subunit. Data presented is from a single representative experiment where the cell protein was used for both 5-HT_3 binding assays and assessment of myc-immunoreactivity following SDS-PAGE and western blotting. Specific [^{3}H]granisetron binding (≈3 nM; non-specific binding defined by the inclusion of ondansetron, 10 μM) to the h5-HT_{3A}-myc receptor in the absence of tunicamycin = 2.644 pmol mg^{-1} protein and represents 100% h5-HT_{3A}-myc binding.

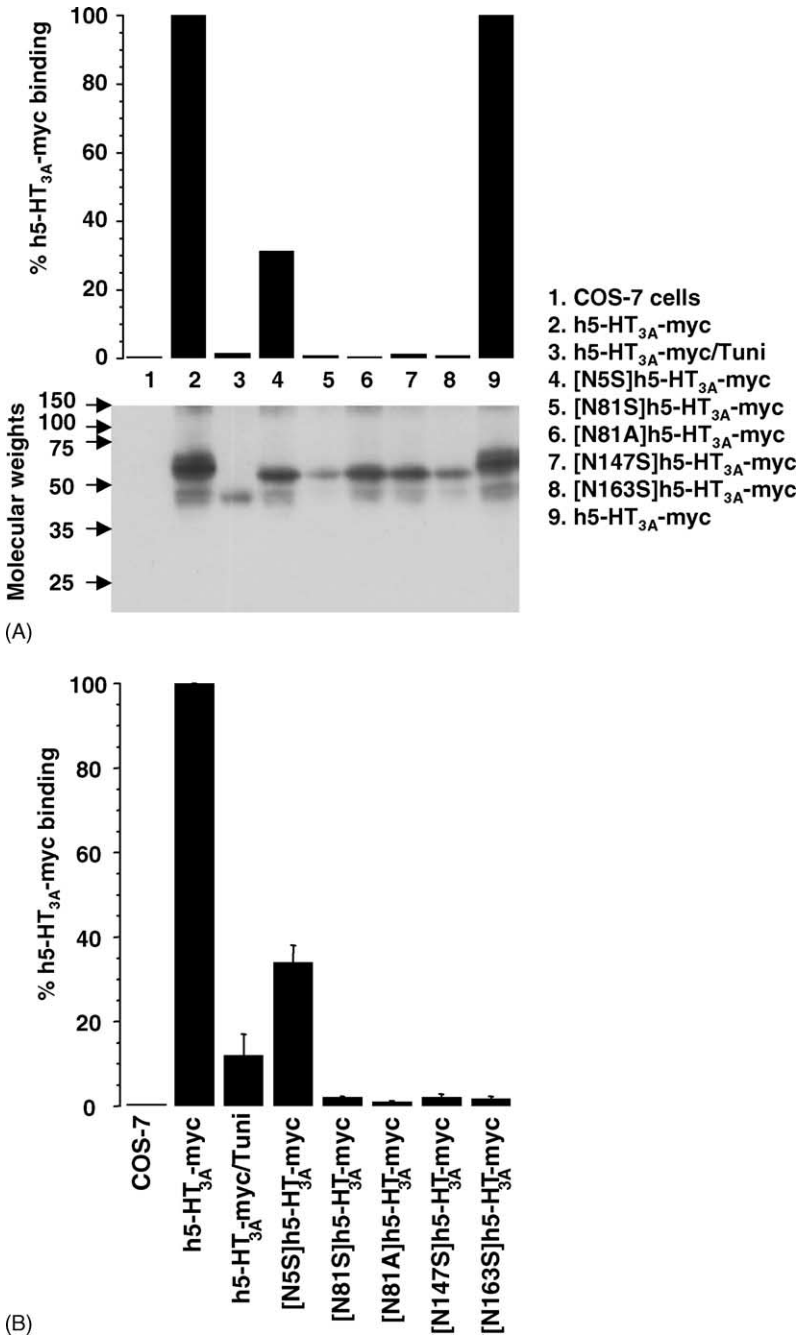

Fig. 4. Consequences of preventing *N*-glycosylation at specific N-terminal asparagine residues in the h5-HT_{3A}-myc subunit. (A) Effect of preventing *N*-glycosylation at each of the four N-terminal asparagine residues on specific [^{3}H]granisetron 5-HT_3 receptor binding (top panel) and apparent molecular weights of myc-immunoreactive protein species (western blot; bottom panel) resulting from COS-7 cells transiently transfected with cDNA encoding the respective h5-HT_{3A}-myc subunit. Site-directed mutagenesis was used to mutate individually each N-terminal asparagine residue (N) to serine (S); N5S, N81S, N147S and N163S. In addition, one asparagine residue was additionally mutated to alanine (A); N81A. Data presented is from a single representative experiment where the cell protein was used for both 5-HT_3 binding assays and assessment of myc-immunoreactivity following SDS-PAGE and western blotting. Specific [^{3}H]granisetron binding (≈11 nM, which is a saturating concentration of the radioligand [16]; non-specific binding defined by the inclusion of ondansetron, 10 μM) to wild-type h5-HT_{3A}-myc receptor in the absence of tunicamycin = 3.742 pmol mg^{-1} protein and represents 100% h5-HT_{3A}-myc binding. (B) Effect of preventing *N*-glycosylation at each of the four N-terminal asparagine residues on specific [^{3}H]granisetron (2.3–5.3 nM) 5-HT_3 receptor binding (non-specific binding defined by the inclusion of ondansetron, 10 μM) resulting from cells transiently transfected with cDNA encoding the respective wild-type or mutant h5-HT_{3A}-myc subunit (h5-HT_{3A}-myc/Tuni; wild-type subunit cDNA transfected into COS-7 cells grown in the presence of tunicamycin). Specific [^{3}H]granisetron binding (non-specific binding defined by the inclusion of ondansetron, 10 μM) to the h5-HT_{3A}-myc receptor in the absence of tunicamycin = 2.006 ± 0.467 pmol mg^{-1} protein and represents 100% h5-HT_{3A}-myc binding. Data represents mean ± SEM, n = 3–5.

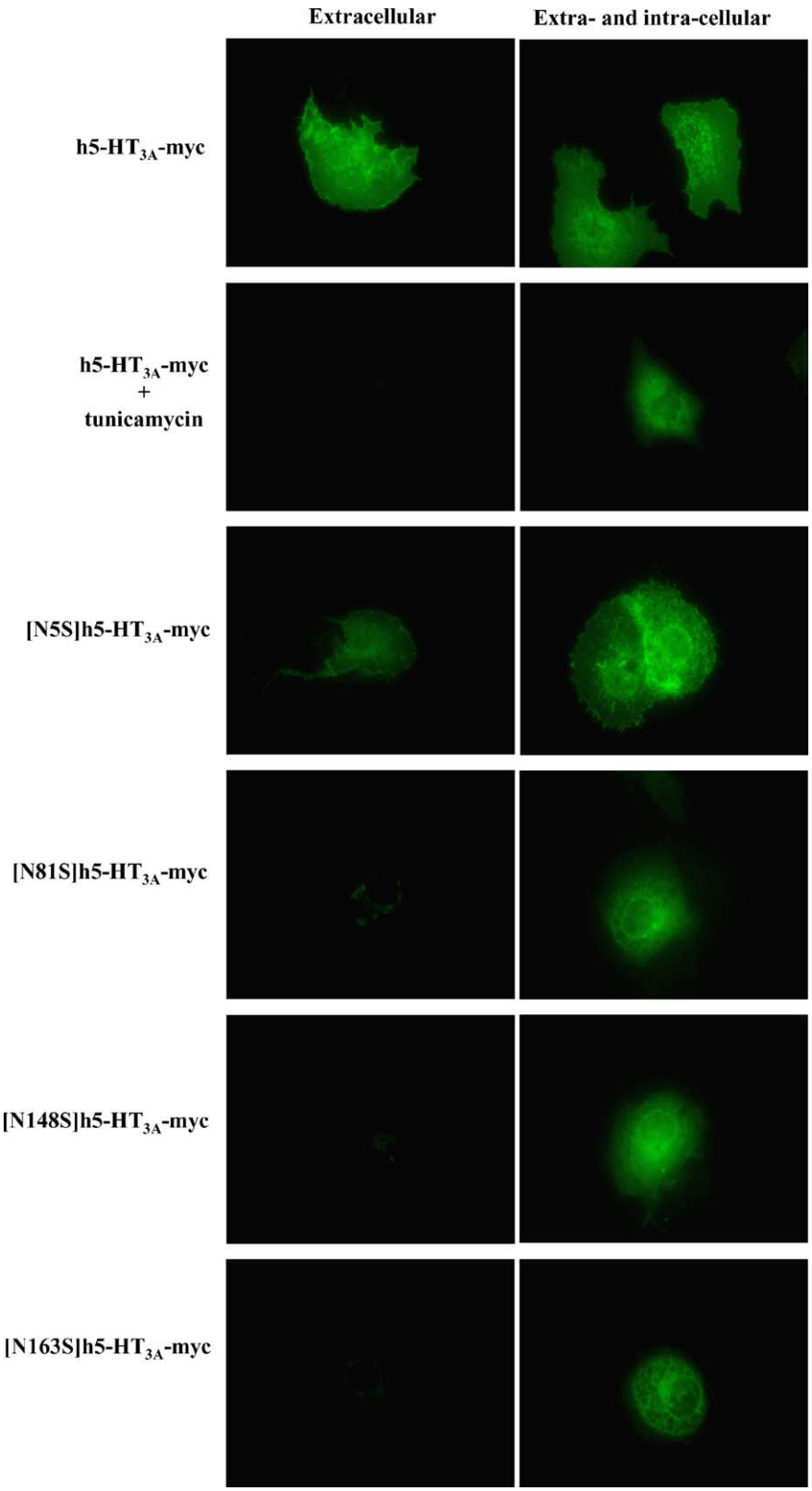

Fig. 5. Effect of tunicamycin treatment (48 h) and N-terminal asparagine residue mutation on the cellular localisation of myc-immunoreactivity in COS-7 cells transiently transfected with either h5-HT_{3A}-myc or various N-terminal asparagine substitution mutants. Extracellular myc-immunoreactivity was assessed in the absence of the detergent, TX100, whilst inclusion of this detergent gave additional antibody access to the intracellular myc-immunoreactivity (extra- and intra-cellular). Cells are representative within a population with independent experiments repeated at least three times. All images were collected with the same exposure time from an individual experiment. Subsequent to the assessment of myc-immunoreactivity with a monoclonal primary antibody (secondary, horse anti-mouse-fluorescein-labelled [green]), all cells were shown to express 5-HT_{3A}-immunoreactivity using a polyclonal (rabbit) antibody, in the presence of TX100, recognising the large intracellular loop of the 5-HT_{3A} subunit ([17] secondary, goat anti-rabbit AlexaFluor 568 [red]; data not shown).

sequence failed to prevent high affinity [^{3}H]granisetron binding. Furthermore, the structurally different selective 5-HT_3 receptor antagonist, ondansetron, and the natural agonist, 5-HT, displayed similar affinity for the [^{3}H]granisetron-labelled binding site formed by h5-HT_{3A}-myc (pIC_{50} = 7.86 ± 0.10 and 6.15 ± 0.12, Hill coefficient = 0.95 ± 0.02 and 1.52 ± 0.10, ondansetron and 5-HT, respectively, mean ± SEM, n = 3; Fig. 2) compared to the wild-type h5-HT_{3A} homomeric receptor (e.g. [21]). COS-7 cells transfected with vector not containing the h5-HT_{3A}-myc insert failed to express specific [^{3}H]granisetron binding (Fig. 3).

Incubation of COS-7 cells transiently transfected with the cDNA encoding h5-HT_{3A}-myc with the *N*-glycosylation inhibitor, tunicamycin (1.0 μg/ml), resulted in a time-dependent reduction in specific [^{3}H]granisetron binding levels (Fig. 3) as well as a time-dependent change in the proportion of different molecular weight proteins displaying myc immunoreactivity (Fig. 3). Thus, in the absence of tunicamycin, h5-HT_{3A}-myc cDNA generated a predominant myc-immunoreactive species with a molecular weight ≈59 kDa with a minor species (often expressed as a doublet, possibly as a consequence of differential post-transcriptional or additional post-translational events) with a molecular weight of ≈45 kDa. However, the presence of tunicamycin resulted in a time-dependent loss of the higher molecular weight myc-immunoreactive species with a corresponding increase in a lower molecular weight myc-immunoreactive species at ≈45 kDa (Fig. 3). COS-7 cells transfected with vector not containing the h5-HT_{3A}-myc insert failed to express either myc- or 5-HT_{3A}-immunoreactivity (Fig. 3; data not shown).

Individual mutation of the four asparagine residues in the extracellular N-terminal domain failed to prevent expression of the mutated h5-HT_{3A}-myc protein (Fig. 4). However, the molecular weights of the predominant species of individual mutants were smaller (by ≈3 kDa) than that observed for the wild type h5-HT_{3A}-myc protein (Fig. 4), but greater than the non-*N*-glycosylated receptor protein expressed in the presence of tunicamycin (1.0 μg/ml; Fig. 4).

In addition to reducing the apparent molecular weights of the mutated protein, individual substitution of the four N-terminal asparagine residues reduced (N5) or abolished (N81, N147, N163) specific [^{3}H]granisetron binding in the resultant COS-7 cell homogenate (Fig. 4).

Immunocytochemical studies also demonstrated that neither tunicamycin treatment, nor individual mutation of the four N-terminal asparagine residues to serine prevented the expression of either myc- (Fig. 5) or 5-HT_{3A}-immunoreactivity (data not shown) by COS-7 cells transiently transfected with the appropriate cDNA. However, comparison of the myc-immunoreactivity evident in the absence and presence of the detergent, TX100, demonstrated that the tunicamycin treatment, and three of the four individual asparagine-to-serine mutations (N81S, N147S and N163S) almost completely prevented cell surface expression of h5-HT_{3A} receptor (Fig. 5). In contrast, the remaining mutant ([N5S]h5-HT_{3A}) was readily apparent in the cell membrane (Fig. 5), although qualitative comparison with the relative level of wild-type h5-HT_{3A}-myc protein in the cell membrane indicated a considerable reduction in the relative proportion of [N5S]h5-HT_{3A} protein expressed in the cell membrane (Fig. 5).

4. Discussion

To facilitate the present studies, we generated a myc-tagged h5-HT_{3A} subunit. The myc-epitope was inserted into the N-terminal mature peptide sequence such that the epitope would reside in the extracellular matrix upon correct insertion of the subunit into the cell membrane, allowing antibody access to the myc-epitope in intact cells, i.e. non-permeabilised cells. Since the present studies used radioligand binding as one method to quantitate 5-HT_3 receptor expression, it was important that the h5-HT_{3A}-myc subunit generated binding sites that retained high affinity for the [^{3}H]-derivative of the selective 5-HT_3 receptor antagonist, granisetron. In addition, the affinities of the structurally unrelated selective 5-HT_3 receptor antagonist, ondansetron, and the natural agonist, 5-HT, for the [^{3}H]granisetron-labelled h5-HT_{3A}-myc receptor were consistent with their respective affinities for the wild-type h5-HT_{3A} receptor [21]. Furthermore, the ability of 5-HT to compete with positive cooperativity (high Hill coefficient) for the [^{3}H]granisetron-labelled h5-HT_{3A}-myc binding site further supports the formation of a multimeric h5-HT_{3A}-myc receptor complex. These findings are consistent with a previous study, which also inserted the myc-epitope into the N-terminus of the h5-HT_{3A} subunit [22]; although the location of the myc-tag selected in this previous study removed one of the four consensus sequences for *N*-glycosylation in the N-terminus (N5 in the present study).

Also consistent with the study of Boyd et al. [22], incubation of cells transfected with h5-HT_{3A}-myc cDNA with the antibiotic, tunicamycin, reduced the estimated molecular weight of the immunoreactive h5-HT_{3A}-myc protein. Tunicamycin inhibits *N*-glycosylation of proteins by preventing the first step in the formation of the oligosaccharide prior to transfer to an appropriate asparagine residue within the polypeptide backbone. These studies suggest that the h5-HT_{3A} subunit is subject to *N*-glycosylation. Indeed, whilst tunicamycin did not prevent h5-HT_{3A}-myc protein expression, the absence of a 5-HT_{3A} binding site suggests an important role for glycosylation in the generation or stability of mature functional 5-HT_{3A} receptors. A further report described a similar role for glycosylation of murine 5-HT_{3A} subunits

expressed in insect cells using the baculovirus system [23]. However, since tunicamycin will inhibit *N*-glycosylation of all cellular proteins, this may interfere with the processes responsible for the correct folding and assembly of multimeric 5-HT_3 receptors. Therefore, in an attempt to more specifically assess the presence and role of *N*-glycosylation of the h5-HT_{3A} subunit, we used site-directed mutagenesis to individually mutate relevant asparagine residues in the h5-HT_{3A}-myc subunit amino acid sequence and hence identify whether *N*-glycosylation occurred at specific asparagine residues and the subsequent consequences of preventing this *N*-glycosylation.

We have identified that each of the four putative *N*-glycosylation sites (NXS/T; Fig. 1) in the extracellular N-terminus of the h5-HT_{3A} receptor subunit is capable of glycosylation when this subunit is expressed heterologously in COS-7 cells. Thus, individual mutation of each N-terminal asparagine (N5, N81, N147, N163) resulted in a reduction in the estimated molecular weight (≈3 kDa) of the predominant protein species. Further support that *N*-glycosylation occurs at multiple asparagine residues within the h5-HT_{3A} receptor subunit was evident from the failure of individual asparagine mutations to reduce the estimated molecular weight to such an extent as incubation with tunicamycin. In fact, the utilisation of all four predicted *N*-glycosylation sites could be implied from the combined reductions in the molecular weights of each mutant (≈3 kDa × 4 = ≈12 kDa) which approximates to the reduction in the molecular weight of the predominant myc-immunoreactive species induced by the presence of tunicamycin (≈59–45 kDa). The estimated sizes of these protein species being consistent with the predicted molecular weight of the mature h5-HT_{3A}-myc protein (54 kDa).

5-HT_3 receptor radioligand binding studies with homogenate preparations from each of four expressed mutants indicated that prevention of *N*-glycosylation interfered with the formation of a 5-HT_3 receptor binding site. Thus, in three of the four mutants ([N81S]h5-HT_{3A}-myc, [N147S]h5-HT_{3A}-myc and [N163S]h5-HT_{3A}-myc, and also a further mutant where N81 was mutated to alanine, [N81A]h5-HT_{3A}-myc, which, relative to [N81S]h5-HT_{3A}-myc, displayed higher levels of protein expression similar to the levels of the other *N*-glycosylation site-directed mutants) specific radioligand binding was negligible, and binding was much reduced in the remaining mutant ([N5S]h5-HT_{3A}-myc). The negligible 5-HT_3 receptor binding apparent with three of the expressed mutants may reflect a greatly reduced affinity of the radioligand, [^{3}H]granisetron, for the mutated protein. However, binding studies were also performed at a saturating radioligand concentration ≈5 times the Kd value (≈11 nM (e.g. [16]), to rule out the presence of 5-HT_3 receptor binding sites with a modest reduction in affinity for the radioligand. Furthermore, the similar percentage reduction in 5-HT_3 receptor binding to the [N5S]h5-HT_{3A}-myc mutant at both radioligand concentrations (≈Kd and ≈5 × Kd) indicates that the affinity of the radioligand was similar for both the wild-type and [N5S]h5-HT_{3A} mutant.

It was interesting that the results of the immunocytochemical studies mirrored the radioligand binding studies. Thus, three of the four mutants ([N81S]h5-HT_{3A}-myc, [N147S]h5-HT_{3A}-myc and [N163S]h5-HT_{3A}-myc) were virtually absent from the cell membrane, with protein expression occurring predominantly within the intracellular compartment. Indeed, the intracellular lattice-like pattern frequently observed with the expressed mutants, and the wild-type h5-HT_{3A}-myc subunit expressed in the presence of tunicamycin, suggests that the immunoreactive proteins were predominantly associated with the endoplasmic reticulum, as has been suggested previously for 5-HT_3 receptor subunits expressed by cells grown in the presence of tunicamycin [22].

The lack of specific [^{3}H]granisetron binding to three of the four mutants (N81, N148 and N163), and the corresponding relative absence of membrane expression, may be interpreted in a number of ways. Clearly individual *N*-glycosylation at these three N-terminal asparagine residues would appear to be critical for the formation of a ligand binding site recognising the selective 5-HT_3 receptor radioligand, [^{3}H]granisetron. However, it may also indicate that lack of *N*-glycosylation at one of these three sites reduces the stability of the homomeric 5-HT_{3A} receptor complex leading to a reduced expression of the receptor in the membrane and the formation of a ligand binding site. Indeed, this is consistent with the long held view that *N*-glycosylation of proteins confers conformational stability and protease resistance (e.g. [24]). However, since qualitative assessments of the expression of each mutant by either Western blot analysis or immunocytochemistry (permeabilised cells) revealed that there was not such a correspondingly large reduction in mutant protein expression relative to the negligible levels of specific 5-HT_3 receptor binding or membrane protein expression, this suggests that substantial degradation of the subunit was not primarily responsible for the relative absence of membrane expression and formation of a ligand binding site.

Given the absence of 5-HT_3 receptor binding and membrane expression following mutation of either N81, N147 or N163, but the less dramatic reduction in binding and membrane expression following mutation of N5, it is of interest that the three 'critical' *N*-glycosylation sites are either within, or less than five amino acids away from, the corresponding regions of the ligand binding loops (A–F) identified in proteins recognising acetylcholine (nicotinic acetylcholine receptor, acetylcholine binding protein (e.g. see [25]) which have been used as a template to model the ligand binding regions of the 5-HT_3 receptor (e.g. [26]). Hence, local structural modification (e.g. prevention of *N*-glycosylation) is likely to have a dramatic effect on the construction of

the 3-dimensional ligand recognition site that is consistent with the relative absence of radioligand binding to these three mutants. In contrast, at least in terms of primary structure, N5 is relatively distant from any of the predicted binding loops (over 50 amino acids) which may correlate with the more modest effect on radioligand recognition following prevention of *N*-glycosylation at this latter site.

It may also be noteworthy that the conservation of the consensus *N*-glycosylation sites across species appears to reflect the apparent significance of the individual *N*-glycosylation sites. Thus, canonical *N*-glycosylation motifs corresponding to the three crucial extracellular *N*-glycosylation sites (i.e. in human N81, N148 and N163) are present in all species identified to date (human ^{81}N-I-T, mouse N-V-T, rat N-V-T, guinea-pig N-I-T, ferret N-I-T; human ^{148}N-C-S, mouse N-C-S, rat N-C-S, guinea-pig N-C-S, ferret N-C-S; human ^{163}N-I-S, mouse N-I-T, rat N-I-S, guinea-pig N-I-S, ferret N-I-S [27–31]), whereas in contrast, *N*-glycosylation motifs corresponding to the N5 *N*-glycosylation site in human are absent in the 5-HT_{3A} subunit amino acid sequence derived from mouse and rat, although canonical motifs are present in ferret (–NSS–) and guinea-pig (–NST–) sequences.

In summary, we have demonstrated for the first time that each of the four N-terminal putative *N*-glycosylation sites in the h5-HT_{3A} receptor subunit is capable of being *N*-glycosylated. Furthermore, preventing *N*-glycosylation at any of these four sites either reduces, or essentially abolishes, membrane expression of the protein and the formation of a 5-HT_3 receptor binding site.

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