

Post-Translational Modifications of the Serotonin Type 4 Receptor Heterologously Expressed in Mouse Rod Cells

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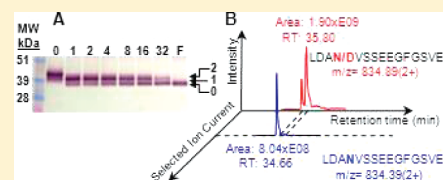
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S Supporting Information

ABSTRACT: G-Protein-coupled serotonin receptor type 4 (5-HT₄R) is a pharmacological target implicated in a variety of gastrointestinal and nervous system disorders. As for many other integral membrane proteins, structural and functional studies of this receptor could be facilitated by its heterologous overexpression in eukaryotic systems that can perform appropriate post-translational modifications (PTMs) on the protein. We previously reported the development of an expression system that employs rhodopsin's biosynthetic machinery in rod cells of the retina to express heterologous G-protein-coupled receptors (GPCRs) in a pharmacologically functional form. In this study, we analyzed the glycosylation, phosphorylation, and palmitoylation of 5-HT₄R heterologously expressed in rod cells of transgenic mice. We found that the glycosylation pattern in 5-HT₄R was more complex than in murine and bovine rhodopsin. Moreover, overexpression of this exogenous GPCR in rod cells also affected the glycosylation pattern of coexisting native rhodopsin. These results highlight not only the occurrence of heterogeneous PTMs on transgenic proteins but also the complications that non-native PTMs can cause in the structural and functional characterization of both endogenous and heterologous protein targets.



G-Protein-coupled receptors (GPCRs) are versatile biological sensors. They are pivotal regulators of cellular responses to a wide spectrum of hormones and neurotransmitters and are involved in a broad range of sensory physiology, including sight, smell, and taste.¹ In mammals, the 5-hydroxytryptamine (5-HT, serotonin) family of receptors (5-HTRs) has been implicated in a variety of neurological and systemic functions, including modulation of memory, aggression, appetite, sexuality, sleep, cognition, thermoregulation, perception, reward, anger, and mood.^{2,3} 5-HT₄R also could serve as a target for the development of new drugs for the treatment of Alzheimer's disease, congestive heart failure, opioid-induced respiratory depression, feeding-associated diseases such as anorexia, and major depressive disorders and are the targets of drugs for the treatment of gastrointestinal diseases such as chronic idiopathic constipation.^{3,4}

Most GPCRs are naturally expressed at such low levels, rhodopsin constituting a notable exception, that heterologous expression systems must be used to obtain sufficient material for their biophysical characterization. In vitro eukaryotic cell systems are most often employed for this purpose because they can perform the complex post-translational modifications (PTMs) required for efficient membrane targeting, stability, and function.

With improved detection technologies, the list of protein modifications reported has increased to more than 300.^{5,6} Some PTMs, such as phosphorylation, are transient even though they play essential roles in intracellular signaling. Others, including glycosylation, lipidation, and disulfide bridge formation, are

more stable, and these are important for maturation and proper folding of newly synthesized proteins.⁷ N-Glycosylation is one of the most common forms of post-translational modification, and it is intricately involved in various cellular processes, including protein folding, protein secretion, intracellular trafficking, stability, binding affinity, enzyme activity, and substrate specificity, allowing the fine-tuning of a protein's function.⁸

Heterogeneity of its PTMs can interfere with the function, stability, and/or crystallizability of a recombinant protein. Homogeneity of a protein population used for crystallization campaigns is usually judged by the sharpness of its electrophoretic band, heterogeneous glycosylation being the main cause of band smearing. For this reason, proteins destined for crystallization trials are often enzymatically or mutationally deglycosylated. Size-exclusion chromatography (SEC) is used routinely to judge sample oligomerization and/or polydispersity, but the resolution of SEC is generally not sufficient to separate different post-translationally modified protein species (with the possible exception of hyperglycosylated proteins). In addition, many other PTMs causing population heterogeneity that can be potentially detrimental for expression, functional characterization, or crystal growth are not evident on

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sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels. In these cases, more laborious techniques or strategies are needed to detect and eliminate population heterogeneity.⁶

Our laboratory has developed an *in vivo* system for the expression of GPCRs in rod photoreceptors of *Xenopus*⁹ and mice.^{10,11} This system was validated with tens of different GPCRs, co-expressed as a transgene along with rhodopsin in retinal rod cells. Characterization of four of these recombinant GPCRs [adenosine A1 receptor (A_{A1}R), 5-HT₄R, 5-HT_{1A}R, and sphingosine 1-phosphate receptor 1] revealed that they were produced in a pharmacologically relevant conformation and that their glycosylation pattern was more homogeneous than when they were expressed in a mammalian cell culture.

In this work, we further examined the PTMs of 5-HT₄R expressed in mouse rod cells with the aim of minimizing protein heterogeneity prior to embarking upon crystallization trials. Our analysis indicated that PTMs of 5-HT₄R were heterogeneous when expressed in this system. We also analyzed murine rhodopsin for comparison and found that its glycosylation pattern was more heterogeneous in the presence of co-expressed 5-HT₄R. These results shed light on the biosynthesis and processing of GPCRs both in rod cells specifically and in other heterologous expression systems in general and highlight the often unaddressed occurrence of such non-native PTMs in recombinant proteins.

■ EXPERIMENTAL PROCEDURES

Transgenic Mice. Generation of 5-HT₄R Transgenic (TG) mice was described in detail previously.^{10,11} In brief, the recombinant vector used to generate this TG mouse line contained the mouse rhodopsin promoter, followed by the full-length coding sequence for human 5-HT_{4b}R and the immunopurification tags T7 (MASMTGGQQMG) and Rho15 (C-terminus of rhodopsin). The recombinant expression construct was microinjected into 18-hour-old hybrid C57BL/6J and FVB/NJ embryos (Taconic, Germantown, NY) that were then implanted into pseudopregnant female mice to produce the founder stock. To minimize random insertion of the artificial transgene, interference with other gene(s), or vice versa, we first selected founders on the basis of the highest level of expression of the transgene by their offspring. Then we determined by gel electrophoresis and immunohistochemistry that 3-week-old mice exhibited the highest levels of expression of 5-HT₄R.

Purification of 5-HT₄R and Rhodopsin. For the purification of 5-HT₄R, eyes were enucleated from 3-week-old offspring of TG and WT mice and then frozen. Typically, 4000–6000 frozen eyes were ground to a fine powder with a mortar and pestle under liquid nitrogen. All the subsequent steps were performed at 4 °C. The frozen powder was transferred to a Dounce tissue grinder and homogenized further in 20 mM Tris buffer (pH 7.4) (with protease inhibitors, 10 µg/mL benzamidine and 1 mM phenylmethanesulfonyl fluoride). The concentration of the 5-HT₄R antagonist, GR125487, was kept at 250 nM at all times during the purification, except during receptor centrifugal concentration when the concentration was increased to avoid ligand depletion. The suspension was centrifuged for 30 min at 48400g and 4 °C, and the supernatant was discarded. The membrane pellet was resuspended in 1 L of TBS [50 mM Tris (pH 7.4), 280 mM NaCl, and 6 mM KCl] and solubilized with 0.1% (w/v) *n*-dodecyl β -D-maltoside (DDM) and 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) with rotation for 3–4 h. Insoluble

material was removed by centrifugation (45 min at 8700g) and filtration through paper. The supernatant was incubated with slow rotation overnight with 13 mL of agarose-immobilized T7 antibody (Novagen, Madison, WI) and then loaded onto a column. The T7 column was washed with 200 mL of washing buffer (1 mM DDM in TBS) and eluted overnight with 200 mL of T7 competing peptide (MASMTGGQQMG) in washing buffer. Immediately before this elution, a 3.5 mL column with agarose-immobilized 1D4 monoclonal antibody¹² was coupled in tandem to the T7 column to capture the eluted receptor. After elution, the T7 column was removed, and the 1D4 resin was washed and eluted with 1D4 competing peptide (TETSQVAPA) in washing buffer. The eluate was concentrated to 1 mL and loaded in two runs onto a SEC Superdex 200 column (GE Healthcare, Piscataway, NJ) equilibrated with washing buffer. The resulting fractions containing 5-HT₄R were concentrated with 50 kDa centrifugal concentrators to 1–2 mg/mL and separated in NuPAGE 4 to 12% polyacrylamide SDS–PAGE gels (Invitrogen, Carlsbad, CA). Receptor bands were cut out from gels after they had been stained with Coomassie G-250 (SimplyBlue SafeStain, Invitrogen) and used for mass spectrometry (MS) analyses. The typical yield for a preparation of this scale was ~1 mg of purified 5-HT₄R.

WT mouse rhodopsin from TG mice expressing 5-HT₄R was purified from the flow-through of the T7 column after the first step of 5-HT₄R purification (this contained just trace amounts of 5-HT₄R but all the mouse rhodopsin). Rhodopsin was purified with a 1D4 column and Superdex 200 column in a manner similar to that used for 5-HT₄R.

Rhodopsin from WT mice was purified from 22 WT mouse eyes. Eyes were homogenized in a glass Dounce tissue grinder, and the sample was treated in a manner similar to that used for 5-HT₄R, except that the T7 purification step was omitted. Bovine rhodopsin was purified from retinas in a manner similar to that used for mouse rhodopsin except that rod outer segments (ROS) were isolated from dark-adapted bovine retinas¹³ before detergent solubilization.¹⁴

For polysaccharide analyses, ~10% of a purified sample (5-HT₄R or rhodopsin) was incubated with PNGase F prior to SEC, and the resulting preparation was used as a deglycosylated control. For phosphorylation analyses, all 5-HT₄R samples were deglycosylated. To obtain the corresponding dephosphorylated control sample, we treated ~10% of the purified 5-HT₄R with mouse protein phosphatase PP2A.¹⁵

Mass Spectrometry. Bands from SDS–PAGE gels containing 5-HT₄R were subjected to in-gel digestion with either sequencing grade modified trypsin (Promega, Madison, WI) or chymotrypsin (Roche, Indianapolis, IN) according to the procedure described previously.¹⁶ Briefly, pieces excised from a SDS–PAGE gel were first destained in 50% acetonitrile containing 50 mM ammonium bicarbonate and then dehydrated with acetonitrile. Before an overnight proteolytic digestion, proteins were reduced with 20 mM DTT at room temperature for 1 h and alkylated with 50 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 min in the dark. After proteolytic digestion, peptides were extracted from the gel with 5% formic acid in 50% acetonitrile and then resuspended in 0.1% formic acid after being dried completely under vacuum. Identification of 5-HT₄R phosphorylation sites was facilitated by removal of sugar chains from the protein with PNGase F, as well as by enrichment of phosphopeptides with a MonoTip TiO column (GL Science Inc., Tokyo, Japan) according to the manufacturer's protocol. Liquid chromatography–tandem mass

spectrometry (LC–MS/MS) analysis of the resulting peptides was performed with a LTQ Orbitrap XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Reverse-phase high-performance liquid chromatography (HPLC) was conducted with an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA) equipped with a Dionex C18 Acclaim PepMap 100 column (0.075 mm × 150 mm). Mass spectra were recorded by using alternating full and MS/MS scans of the five most abundant precursor ions at the normalized collision energy of 30%. Mass spectrometric data were analyzed with Mascot Daemon (version 2.3.0, Matrix Science, London, U.K.). Phosphorylation of Ser, Thr, and Tyr residues, palmitoylation of Cys residues, carbamidomethylation of Cys residues (because of the iodoacetamide treatment), and oxidation of Met residues were set as variable modifications. Phosphorylation sites suggested by the MASCOT search were verified by manually examining each tandem mass spectrum of phosphopeptides. For the glycosylation study, candidate N-glycosylated sites were initially identified with glycan-free peptides on the basis of the conversion of Asn residues to Asp by PNGase F. Sugar compositions of glycopeptides were verified by manual interpretation of the obtained tandem mass spectra.

For glycosylation analyses, murine and bovine rhodopsins were treated like 5-HT₄R.

RESULTS

Different heterologous expression systems have been shown to introduce varying PTMs in the same protein. For example, rhodopsin is heavily and heterogeneously glycosylated when expressed in HEK293 and COS-1 cells,¹⁷ yeast,¹⁸ NIH 3T3 cells (this work), and mouse liver,¹¹ in contrast to a more sparse and homogeneous glycosylation pattern seen in its native retinal tissue.¹⁹

To investigate this phenomenon, we conducted a detailed comparative analysis of PTMs present in recombinant 5-HT₄R expressed in rod cells versus 5-HT₄R expressed in other systems and included rhodopsin as a reference GPCR. On the basis of amino acid sequence, PTMs predicted for 5-HT₄R were S-palmitoylation at C³²⁸ and N-linked glycosylation at N⁷ and N¹⁸⁰.²⁰ A disulfide bridge between C⁹³ and C¹⁸⁴ was also predicted on the basis of homology to other GPCRs²¹ and experimental data.²² Residues T²¹⁸, T²⁴⁸, and S³¹⁸ are potential protein kinase C phosphorylation sites, and the C-terminus is rich in serine and threonine residues, some of which are potential phosphorylation sites for G-protein-coupled receptor protein kinases.^{20,23}

N-Glycosylation of 5-HT₄R. There are two putative N-linked glycosylation sites on the extracellular side of 5-HT₄R that conform to the consensus sequence N-X-S/T, where X can be any amino acid except a Pro residue. These are located on the extracellular side of 5-HT₄R (N⁷ in the N-terminus and N¹⁸⁰ in the extracellular loop E-II) (Figure 1). Initial electrophoresis in 12% polyacrylamide gels showed a single band for 5-HT₄R expressed in rod cells of TG mice,¹⁰ and SEC on Superdex 200 revealed a sharp, single major peak (Figure S1 of the Supporting Information). Further analysis in 4 to 12% acrylamide NuPAGE gradient gels (Invitrogen) revealed a minor band with a mobility slightly higher than that of the main receptor band (Figure 2A, lane 0). A time course deglycosylation with PNGase F, followed by SDS–PAGE and immunoblotting, showed that both bands collapsed into a new band with a higher mobility, suggesting that the bands correspond to di-, mono-, and deglycosylated species, from top to bottom,

respectively (Figure 2A). The relative intensity of the bands before PNGase F treatment indicated that one site was glycosylated in ~75% of the species (site 1) and the second site in nearly 100% (site 2). Site 1 was easily accessible to PNGase F, as deglycosylation was complete in a few seconds, whereas removal of glycan from site 2 was slow even at room temperature.

By comparison, 5-HT₄R expressed in mouse rod cells was glycosylated more homogeneously than 5-HT₄R expressed in the mammalian cell lines HEK293 and TRex but like the receptor expressed in Sf9 cells, as assessed by electrophoresis (Figure S2 of the Supporting Information). We did not determine which 5-HT₄R residues are glycosylated in insect cells, but because deglycosylation proceeded relatively rapidly and quantitatively, we speculate that only site 1 is glycosylated.

To confirm the electrophoretic results obtained for 5-HT₄R expressed in mouse rod cells, we conducted a detailed MS analysis of 5-HT₄R purified by sequential T7 and 1D4 immunochromatography. The purified receptor was separated by SDS–PAGE to allow independent analysis of monomers and dimers because, as with other GPCRs, receptor oligomerization is often visible in electrophoresis gels. Monomer and dimer bands were cut out and treated separately with in-gel trypsin or chymotrypsin digestion, but both bands produced identical results. The overall peptide coverage for 5-HT₄R in this study was 85% (Figure S3 of the Supporting Information). The glycosylation site N⁷ was identified by a mass increase of 0.9846 Da resulting from transformation of an Asn to an Asp after PNGase F treatment (Figures 2B and 3), as well as by comparing the LC–MS profiles of glycosylated and deglycosylated 5-HT₄R peptides at the same elution time. As shown in Figure 3, the N⁷-containing peptide ⁴LDANVS-SEEGFGSVEK¹⁹ [*m/z* 834.40 (2+)] and its glycoforms were observed within an elution time period of 4 min. After PNGase F treatment, the heterogeneous glycopeptide peaks disappeared and a new *m/z* 834.89 (2+) peak was formed corresponding to the peptide with N⁷ converted to a D residue by PNGase F. Because the free peptide with an ion at *m/z* 834.40 (2+) was also observed in the PNGase F-treated sample, the extent of glycan modification at the N⁷ residue was not 100%. Further quantitative glycosylation mapping was conducted by calculating the ratios of selected ion chromatogram peak areas of converted N/D peptide to total converted and unconverted peptides. It should be noted that deamidation of an Asn residue resulting from a loss of ammonia and formation of an Asp residue causes the exact same mass increase of 0.9846. Thus, the ratio resulting from deamidation in vivo and in vitro must be excluded, which can be estimated from the 5-HT₄R sample not treated with PNGase F. The deamidation ratio of N⁷ was found to be negligible (data not shown), and the glycan occupancy at N⁷ was determined to be ~70% (Figure 2B).

Identification of the glycosylation species present at N¹⁸⁰ was challenging. Neither unmodified nor Asn to Asp converted glycan-free peptides were detected in either glycosylated or deglycosylated 5-HT₄R, suggesting either a site fully occupied by glycans or poor ionization of the peptides. However, glycopeptides consistent with the chymotryptic peptide ¹⁷⁶NQNSNSTYCVF¹⁸⁶ along with various glycan moieties at N¹⁸⁰ were identified with a mass error of <5 ppm by glycopeptide mapping. The tandem mass spectrum of each glycopeptide further confirmed glycan compositions at the specific glycosylation sites (Figure S4 of the Supporting Information). Therefore, the monoglycosylated form of the receptor observed in electrophoresis (Figure 2A) that was slowly deglycosylated

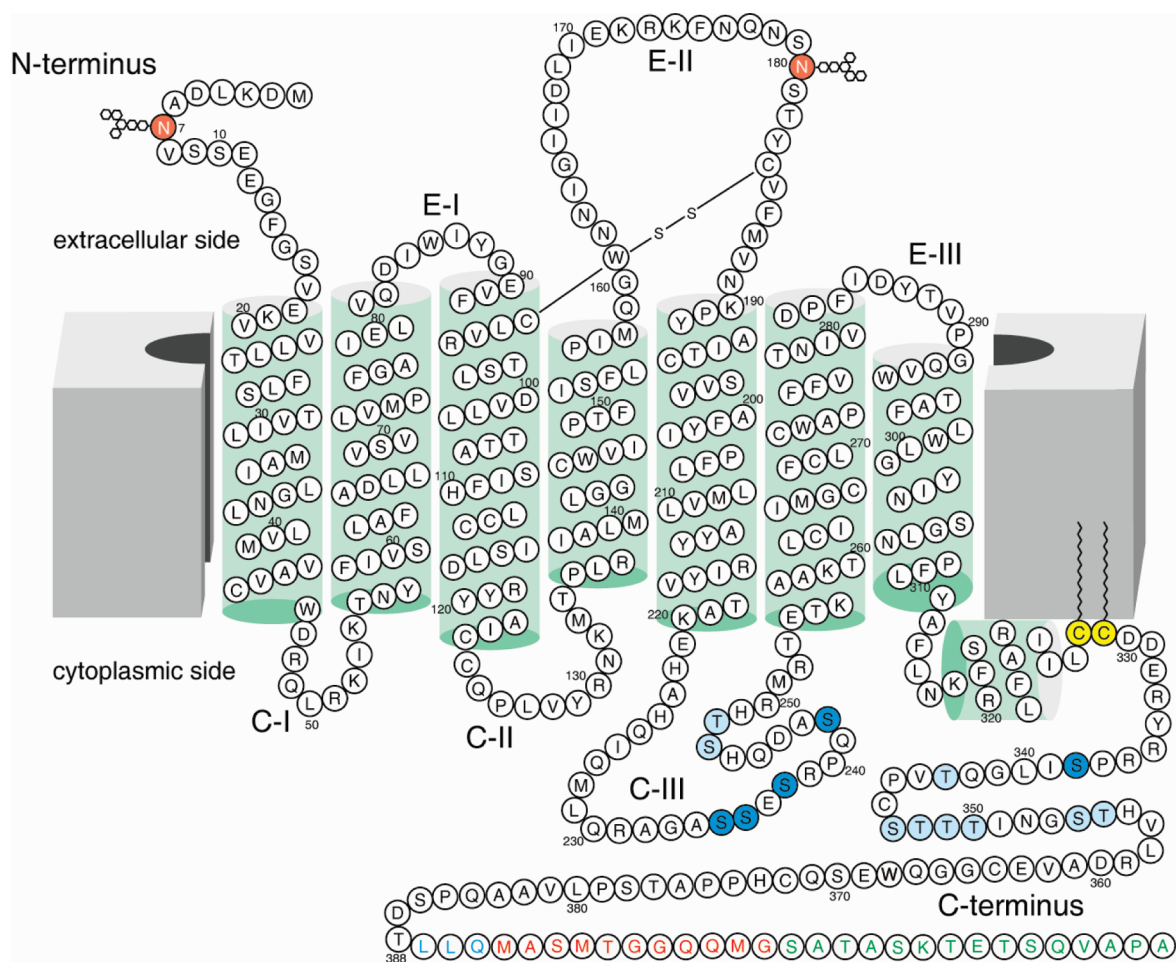


Figure 1. Two-dimensional model of the 5-HT₄R construct used in this work (based on the β 1 adrenergic receptor structure, Protein Data Bank entry 2VT4). Colored circles denote different PTMs found: red for glycosylation, blue for phosphorylation, and yellow for palmitoylation. Light blue circles represent putatively modified residues. A disulfide bridge is predicted on the basis of homology to other GPCRs and experimental evidence. Colored letters indicate the C-terminal linker (blue) and purification tags (red for T7 and green for 1D4 tags).

by PNGase F corresponds to N¹⁸⁰, site 2 in loop E-II, whereas site 1 (easily accessible to PNGase F) corresponds to N⁷ in the N-terminus.

The different glycosylation species identified for 5-HT₄R expressed in the retina of TG mice are summarized in Table 1. High-mannose types and complex types of sugars were identified at both sites. Most of the oligosaccharides had the core structure of three mannose (Man) and two *N*-acetylglucosamine (GlcNAc) residues, Man α 1 \rightarrow 3-(Man α 1 \rightarrow 6)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc \rightarrow Asn [or (Hex)₃(GlcNAc)₂ in the abbreviated nomenclature used in Table 1], common in vertebrate *N*-glycosylated proteins. LC-MS/MS identified a total of 23 species for N⁷ and eight species for N¹⁸⁰, which explains the smearing of the upper band noted in the SDS-PAGE gels (Figure 2A).

The glycosylation pattern observed for 5-HT₄R was unexpectedly complex such that the receptor migrated as two distinct electrophoretic bands (Figure 4, lane 1). In contrast, four other GPCRs, also expressed in TG mouse rod cells (cannabinoid CB2 receptor, AA1AR, 5-HT_{2C}R, and 5-HT₇R), migrated as single electrophoretic bands (Figure S5 of the Supporting Information and ref 11). PNGase F treatment of these receptors showed that they were glycosylated (not shown), but we did not conduct a detailed glycan analysis.

It could be argued that heterogeneity of 5-HT₄R glycans as identified by MS is artifactually influenced by in-source fragmentation. Actually, this method has been used to detect glycopeptides by monitoring low-mass, sugar-specific oxonium ions.^{24,25} However, in-source fragmentation must be specifically enabled. In our experiments, no active source fragmentation was applied. Therefore, heterogeneity of glycans from in-source fragmentation here should be negligible.

To place the glycosylation heterogeneity of 5-HT₄R in TG mice in some perspective, we compared it to co-expressed rhodopsin from the same TG mice. Mouse rhodopsin was purified with an immobilized 1D4 antibody from the flow-through remaining after purification of 5-HT₄R with the immobilized T7 antibody. With this preparation, we then confirmed the presence of analogous glycosylation sites N² and N¹⁵ in mouse rhodopsin and identified the nature of their glycans (Table 1). Again, we were surprised by the large variety of distinct glycosyl species identified (7 for N² and 18 for N¹⁵).

A possible cause of the heterogeneity of rhodopsin glycosylation in TG mice is that 5-HT₄R was being co-expressed with rhodopsin, thereby stressing the PTM machinery in rod cells. Thus, we also analyzed the glycosylation pattern of rhodopsin from WT mice. The results (Table 1) confirm this hypothesis, as the heterogeneity of rhodopsin glycosylation in WT mice was markedly reduced at N¹⁵.

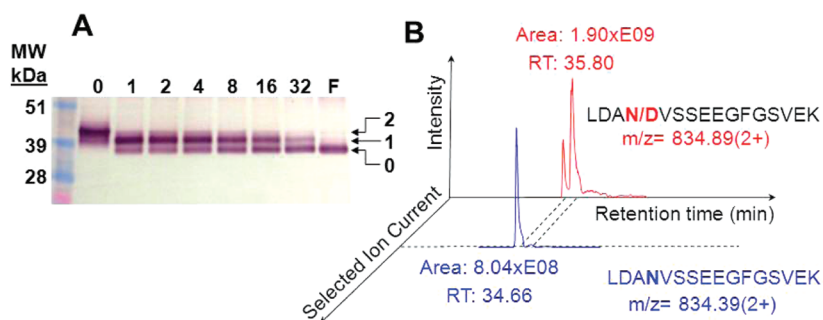


Figure 2. Analysis of 5-HT₄R glycosylation. (A) Time course for PNGase F treatment of 5-HT₄R expressed in mouse rod cells. Numbers above the lanes denote the times in minutes of incubation at room temperature of a DDM extract of transgenic mouse retina. At each time, an aliquot was taken and the deglycosylation reaction was stopped by adding electrophoresis loading buffer. The lane labeled F shows the completed reaction, which was achieved by adding extra PNGase F to an aliquot and incubating the mixture for 1 h at 37 °C. Arrows indicate the three major bands visible in the 1D4 immunoblot of a SDS–PAGE gel, corresponding to diglycosylated, monoglycosylated, and deglycosylated protein, from top to bottom, respectively. The monoglycosylated form corresponds to the receptor glycosylated at N¹⁸⁰ (see the text). (B) Extracted ion chromatogram of ⁴LDANVSSEEGFGSVEK¹⁹ with ions at *m/z* 834.39 (2+) and *m/z* 834.89 (2+). N⁷ was converted to an Asp residue in PNGase F-treated 5-HT₄R.

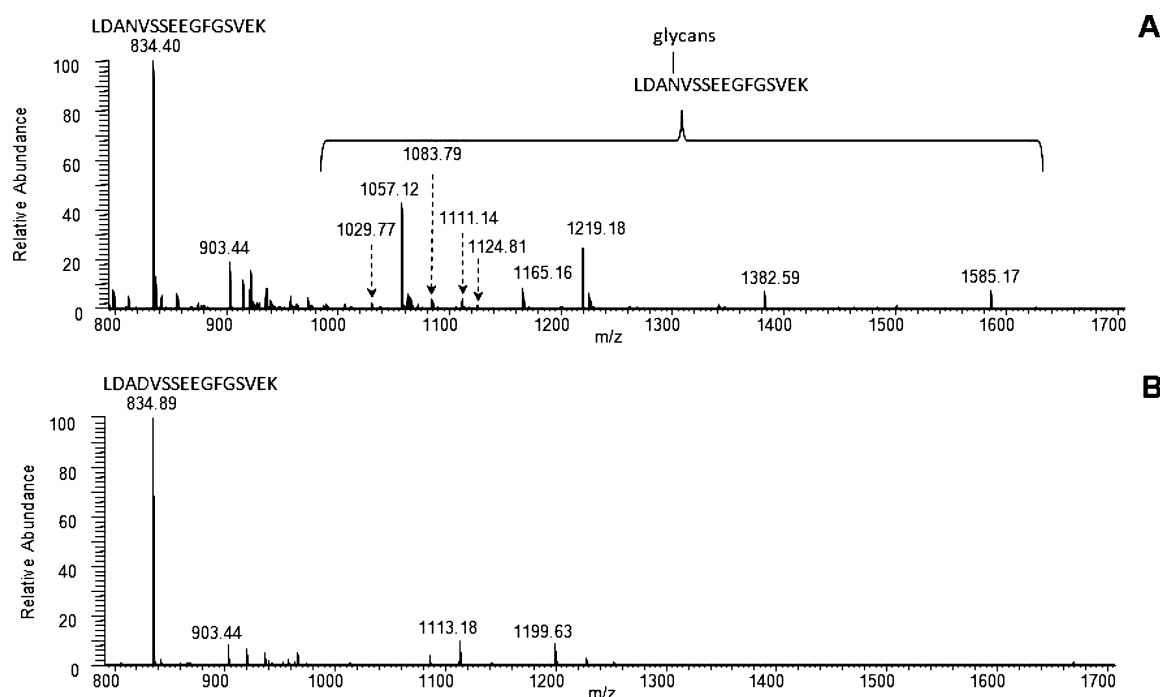


Figure 3. Analysis of 5-HT₄R glycosylation. Mass spectral analysis of chromatographed peptide ⁴LDANVSSEEGFGSVEK¹⁹ samples with ions at *m/z* 834.40 (2+) eluting at 32–36 min: (A) with different glycans and (B) deglycosylated with PNGase F. The peak at *m/z* 834.89 (2+) corresponds to the deglycosylated peptide with a mass shift of 0.980 Da emanating from conversion of the N⁷ to a D⁷ residue after PNGase F treatment.

Nevertheless, a number of distinct glycosylation species were observed by LC–MS/MS for rhodopsin from WT mice. Bovine rhodopsin is known for its homogeneous glycosylation pattern, which has allowed its crystallization in at least five different asymmetric unit arrangements without prior deglycosylation.²⁶ For this reason, we also included bovine rhodopsin in the glycan analysis (Table 1). Eight glycosyl species were found at the N² position and 10 at the N¹⁵ position in bovine rhodopsin. The predominant oligosaccharide form in bovine rhodopsin was GlcNAcβ1→2Manα1→3-(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc→Asn [or (Hex)₁+(Man)₃(GlcNAc)₂ in the nomenclature used in Table 1]. We found that this accounted for ~50% of the glyco species at N¹⁵ in bovine rhodopsin (not shown). This glyco form was previously reported to be the major oligosaccharide structure in bovine rhodopsin²⁷ and accounted

for ~60% of the glyco forms in frog.²⁸ This was also the major oligosaccharide we found at N², but its quantification was complicated because multiple ions were detected for the N²-containing glycopeptide (because of incomplete N-terminal acetylation, oxidation of the M¹ residue, Na⁺ complexes, etc.).

The relative heterogeneity of glycosylation found for the four proteins analyzed by LC–MS/MS (5-HT₄R and rhodopsin in TG mice, rhodopsin in WT mice, and bovine rhodopsin) is consistent with the smeared appearance of electrophoretic bands observed before treatment with PNGase F (Figure 4).

Similar to 5-HT₄R, when we expressed rhodopsin in a mammalian cell culture, a complex hyperglycosylation pattern was found by SDS–PAGE that precluded MS analysis of the individual glycosylated species (Figure S6 of the Supporting Information).

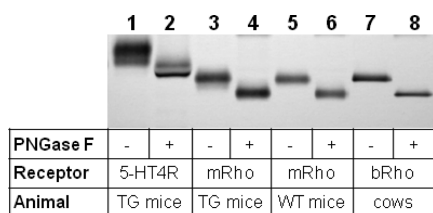


Figure 4. 1D4 immunoblots of purified receptors analyzed in this study, without and with PNGase F treatment: lanes 1 and 2, 5-HT₄R; lanes 3 and 4, rhodopsin purified from 5-HT₄R transgenic mice; lanes 5 and 6, rhodopsin purified from WT mice; lanes 7 and 8, purified bovine rhodopsin. Smearing of glycosylated protein bands parallels the heterogeneity of the glycosylation determined by LC–MS/MS (see Table 1). 5-HT₄R (lane 2) was not completely deglycosylated; the monoglycosylated band is visible above the deglycosylated band.

Phosphorylation of 5-HT₄R. Although Ser, Thr, and Tyr residues typically can be phosphorylated by a variety of cellular kinases, the only major kinase present in rod outer segments is the Ser/Thr protein kinase, GRK1, that specifically phosphorylates activated rhodopsin at three Ser residues in its C-terminus.²⁹ In vivo, 5-HT₄R can be phosphorylated by GRK5 at its C-terminal (S/T residues 347–355) cluster.³⁰ There are 19 potential phosphorylation targets for GRK1 in the intracellular region of 5-HT₄R: five Ser residues and one Thr in loop C-III and six Ser residues and seven Thr residues in the C-terminus (including residues in the purification tags). Because initial experiments in which we treated purified 5-HT₄R with phosphatase PP2A suggested that this receptor was phosphorylated (Figure S7 of the Supporting Information), we analyzed the phosphorylation status of heterologously expressed 5-HT₄R.

LC–MS indicated that a peptide corresponding to the third intracellular loop (A²³²–R²⁵⁰) evidenced highly heterogeneous

phosphorylation (Figure 5 and Table 2). Tandem mass spectra of this peptide revealed the identity of the phosphorylated residues for each species through their cleavage precursors (from the C-terminal and N-terminal directions) (Figure S8 of the Supporting Information). We found that S²³⁵, S²³⁶, S²³⁸, and S²⁴² residues were the targets for phosphorylation in this peptide. For residues S²⁴⁷ and T²⁴⁸ in loop C-III, it was not possible to unambiguously identify which of the two residues was phosphorylated. For Figure 5, the peak area of each extracted ion was calculated. Although the ionization efficiency of phosphorylated and unphosphorylated peptides is different, comparison of the corresponding chromatography areas can provide us some approximate information about their relative abundance. For peptide ²³²AGASSESRPQSADQHSTHR²⁵⁰, the ratio of the unphosphorylated, monophosphorylated, diphosphorylated, triphosphorylated, and tetraphosphorylated species was ~1:47:50:22:3.

Similarly, multiple products with zero to five phosphorylated residues were found for the tryptic peptide R³³⁶–R³⁵⁹ at the C-terminus (Table 2). In this case though, only S³³⁸ was positively identified as being phosphorylated. There are eight possible phosphorylation targets close together in peptide R³³⁶–R³⁵⁹, making it difficult to unambiguously identify individual phosphorylated residues.

Palmitoylation of 5-HT₄R. The MASCOT search of LC–MS/MS data showed a tryptic peptide ³²²AFLILCCDDER³³³ with an ion at *m/z* 853.47 (2+) singly palmitoylated (with the other Cys residue carboxamidomethylated) at position C³²⁸ or C³²⁹ (each predicted to be situated immediately after helix 8). The unpalmitoylated peptide with an ion at *m/z* 762.87 (2+) (both C³²⁸ and C³²⁹ were carboxamidomethylated) was detected around the elution time of 30 min. The addition of a 16-carbon fatty acid to Cys residues turned the peptide into a

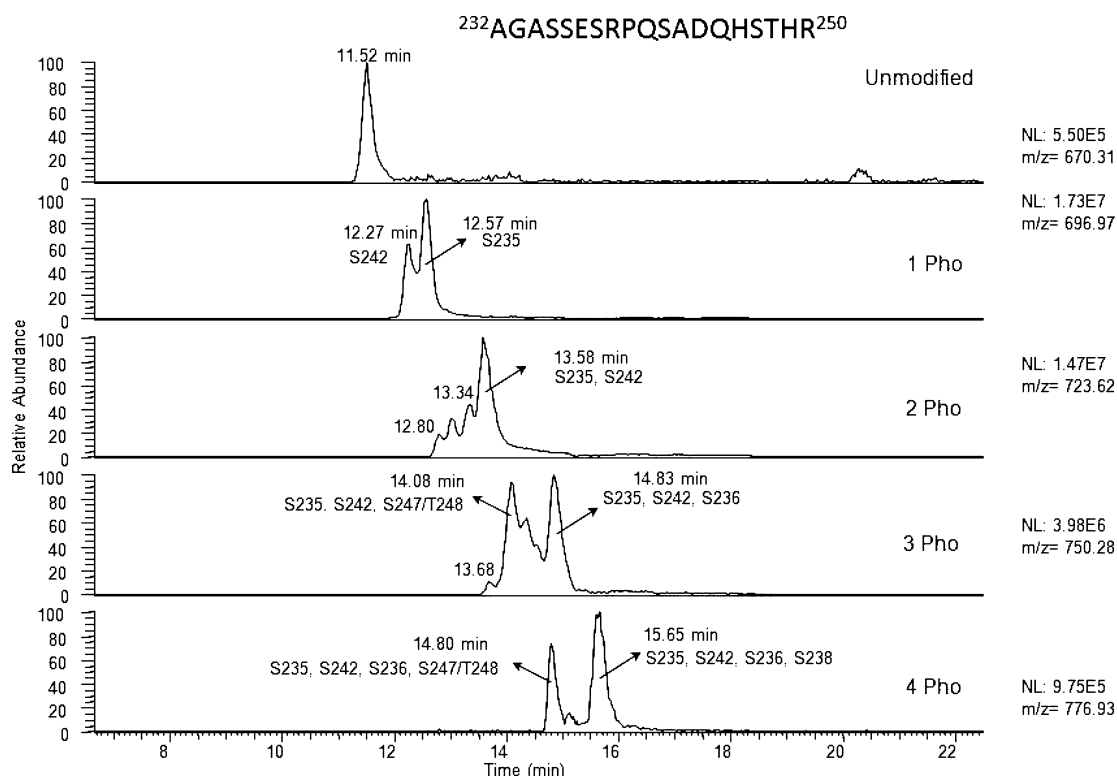


Figure 5. Analysis of 5-HT₄R phosphorylation. Extracted ion chromatography of loop C-III peptide ²³²AGASSESRPQSADQHSTHR²⁵⁰ with zero to four phosphorylated residues. NL stands for normalized peak intensity.

Table 2. Phosphorylation Sites Identified in 5-HT₄R from Transgenic Mice by LC–MS/MS^a

sequence	mass difference	phosphorylation sites
AGASSESRPQ ³²⁸ SADQHSTHR, loop C-III	+1 phosphate	S ²³⁵ or S ²⁴²
	+2 phosphates	S ²³⁵ and S ²⁴²
	+3 phosphates	S ²³⁵ , S ²⁴² , and S ²³⁶ ; S ²³⁵ , S ²⁴² , and S ²⁴⁷ or T ²⁴⁸
	+4 phosphates	S ²³⁵ , S ²⁴² , S ²³⁶ , and S ²³⁸ ; S ²³⁵ , S ²⁴² , S ²³⁶ , and S ²⁴⁷ or T ²⁴⁸
RPSILGQ ³²⁹ TVPCTTTINGSTHVL ³³³ R, C-terminus	+1 phosphate	S ³³⁸
	+2 phosphates	S ³³⁸ and S ³⁵⁴ or T ³⁵⁵
	+3–5 phosphates	S ³³⁸ and 2–4 other residues among T ²⁴³ , S ²⁴⁷ , T ²⁴⁸ , T ²⁴⁹ , T ²⁵⁰ , T ²⁵¹ , S ³⁵⁴ , and T ³⁵⁵

^aThis analysis was performed as described in Experimental Procedures.

very hydrophobic molecule, delaying the elution time more than 25 min. As shown in Figure 6A, there were two separate peaks, 56.91 and 57.77 min, corresponding to the palmitoylated ion at m/z 853.47 (2+), and the area ratio of the two peaks was ~1:5. Hence, it is clear that there were two populations of palmitoylated peptides: one palmitoylated at C³²⁸ and the other at C³²⁹. Further deconvolution of the tandem mass spectrum of

these two elution fractions (at 57.77 and 56.99 min) confirmed the palmitoylation at C³²⁸ and C³²⁹ (Figure 6B,C), respectively. Fragment ions y_5 and b_7 (as well as y_5^* and b_7^*) were critical for this distinction as shown in Figure 6D. Therefore, the ratio of palmitoylation at C³²⁸ to C³²⁹ was ~5:1. A dipalmitoylated form of this peptide was not detected.

Two Cys residues in the peptide ⁹⁷TSLDVLLTTASIFHLC-CISLDR¹¹⁸ were also found to be partially palmitoylated (not shown). Because these residues are located deep within the transmembrane region of helix III, we believe these are chemical modifications that occurred during the treatment of the sample after purification. Alternatively, they could represent an aberrant palmitoylation of misfolded proteins.

DISCUSSION

PTMs are important for biosynthesis and processing, including folding and trafficking, of membrane proteins. Experimentally, highly heterogeneous PTMs can present a considerable barrier to structural elucidation by crystallographic methods because crystal growth requires identical points of interaction between proteins in the crystal lattice. Given that different expression systems (including native expression in different tissues) can produce different PTMs for any given protein, it is prudent to characterize the PTMs involved before embarking on costly structure–function studies. And, as shown in this work, tight

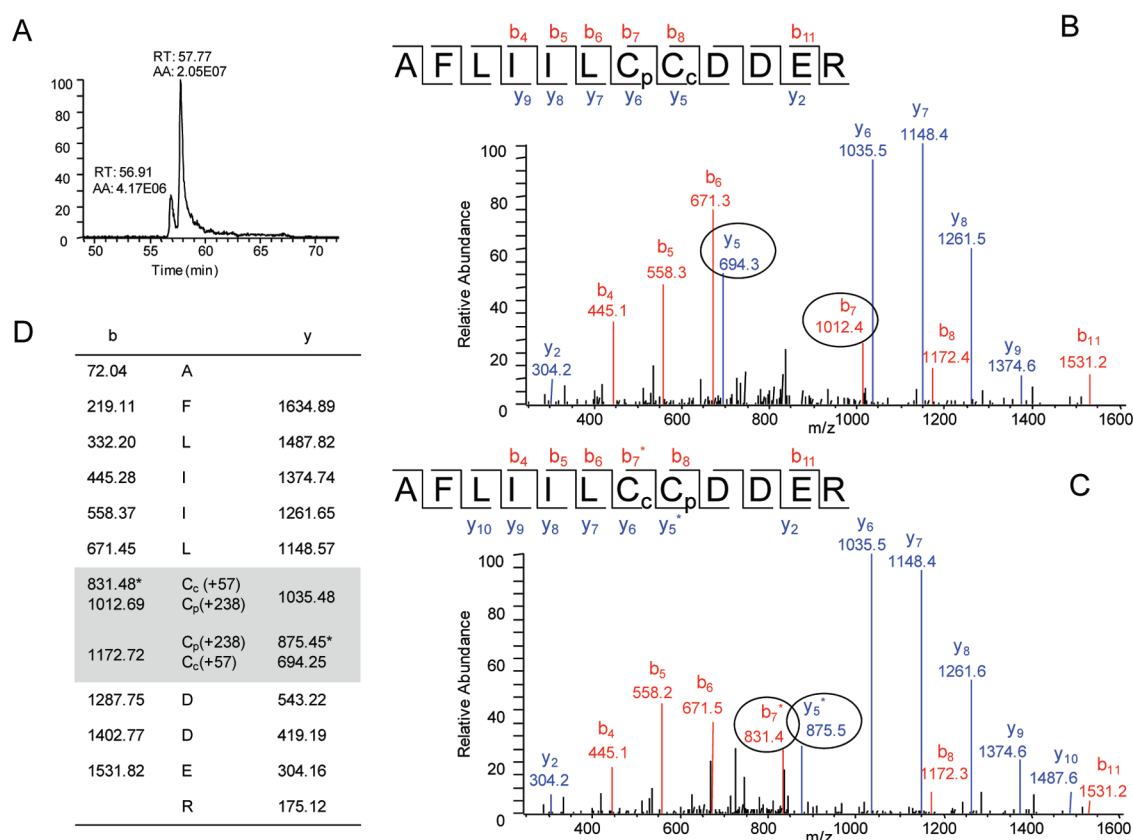


Figure 6. Analysis of 5-HT₄R palmitoylation. (A) Extracted ion chromatogram of the palmitoylated peptide ³²²AFLILCCDDER³³³ with an ion at m/z 853.47 (2+). The mass shift of m/z 295 compared with that of the unmodified peptide corresponds to one Cys palmitoylated (C_p) and the other one carboxamidomethylated (C_c). The deconvoluted tandem mass spectrum of the peptide eluting at 57.77 min was assigned to C³²⁸ palmitoylation (B) and the 56.91 min elution to C³²⁹ palmitoylation (C). This is indicated by fragment ions of m/z 694.3 (y_5) and m/z 1012.4 (b_7) for C³²⁸ palmitoylation (B), as well as m/z 875.5 (y_5^*) and m/z 831.4 (b_7^*) for C³²⁹ palmitoylation (C). (D) Table listing the theoretical mass value of fragment ions corresponding to palmitoylation and carboxamidomethylation at C³²⁸ or C³²⁹. Circled fragment ions show the mass shift of m/z 57.0215 (carboxamidomethylation, C_c) or m/z 238.2297 (palmitoylation, C_p). Parent ions are within 10 ppm for the extracted ion chromatogram.

SDS–PAGE bands widely used to assess homogeneity of a protein sample are poor indicators of PTM homogeneity.

Previously, we reported development of a GPCR expression system in *Xenopus* and murine rod cells and demonstrated that GPCRs can be expressed in a functional form with apparently low heterogeneity (refs 9–11 and this work). Here we present a more detailed MS analysis of 5-HT₄R expressed in mice and show that its glycosylation is in fact quite heterogeneous. In addition, glycans at N^{180;5,26} (in the Ballesteros–Weinstein numbering³¹) proved to be refractory to PNGase F treatment, as revealed by electrophoresis combined with LC–MS/MS. An analogous residue in loop E-II of the β_2 -adrenergic receptor (N^{187;5,26}), which is mutated in all β_2 adrenergic receptor structures reported to date, has also been shown to be inaccessible to PNGase F.³² The distal portion of the β_2 adrenergic receptor E-II loop makes close contacts with the E-I loop,³³ and polysaccharides at N^{187;5,26} may mask a group of aromatic residues in the E-I loop.³⁴ In contrast, N^{154;4,75} located in the proximal portion of the E-II loop of the adenosine receptor 2A E-II loop can easily be enzymatically deglycosylated.³⁵

Our results obtained from TG mice suggest that stresses exerted on the glycosylation machinery due to overexpression of an exogenous GPCR in rod cells may be responsible for the observed increase in glycosylation heterogeneity of endogenous rhodopsin at N¹⁵. It can therefore be supposed that non-native glycosylation of native and recombinant proteins in heterologous expression systems is common.

In any case, bovine rhodopsin is even more homogeneously glycosylated than mouse rhodopsin. Eight glycosylation species were found for N² and 10 for N¹⁵ in bovine rhodopsin, with (Hex)₁+(Man)₃(GlcNAc)₂ accounting for ~50% of the total. In our crystal structure of photoactivated bovine rhodopsin (Protein Data Bank entry 2I37), we were able to partially model the oligosaccharides at both N² and N¹⁵ despite the limited resolution.³⁶ In monomer B, we modeled the linear pentasaccharide GlcNAc-Man-Man-GlcNAc-GlcNAc-N¹⁵. Oligosaccharides at both N² and N¹⁵ are wrapped around the equivalent glycan from another monomer in the same unit cell. In addition, the mentioned pentasaccharide at N¹⁵ from monomer B was found to interact in a head-to-head fashion with the equivalent glycan of monomer B from another unit cell in a manner that could not accommodate a longer oligosaccharide (Figure 3 of ref 36). With this observation in mind, the question of how a heterogeneously glycosylated protein such as bovine rhodopsin can form crystals in which multiple intermolecular glycan–glycan contacts stabilize both the rhombohedral (R32) and trigonal (P3₁12) crystal forms immediately arises.^{26,36} One possible answer could be that minor species incompatible with this arrangement are omitted from the crystal due to steric hindrance. Further speculation is that there is enough room in the solvent cavity of the crystal to allow some flexibility and heterogeneity for branched glycans at N¹⁵ (Figure 3 of ref 36 and Figure 1 of ref 26) (the rhombohedral cell contains ~80% solvent whereas the trigonal crystal form ~71% solvent). Therefore, it seems that complete glycan homogeneity can be helpful but not always absolutely required for protein crystallization, even when these moieties appear favorably positioned to support intermolecular interactions present in some crystal lattices. However, the fact that mouse rhodopsin is glycosylated slightly more heterogeneously than bovine rhodopsin could have made its crystallization more difficult.

We also confirmed that 5-HT₄R heterologously expressed in TG mouse retina was phosphorylated, conforming with reports that activated rhodopsin can enhance GRK1-mediated phosphorylation of exogenous proteins in the retina.³⁷ However, the level and heterogeneity of 5-HT₄R phosphorylation were unexpected because GRK1 (the only GRK present in rod outer segments) phosphorylates only three Ser residues in the C-terminus of rhodopsin in a light-dependent manner²⁹ despite the abundance of Ser and Thr residues in rhodopsin's C-terminus and C-III loop. Consequently, we then routinely treated 5-HT₄R with mouse protein phosphatase PP2A during its purification, to eliminate or minimize the number of phosphorylated residues. MS results also indicate that 5-HT₄R is heterogeneously phosphorylated in the C-III loop and C-terminus when overexpressed in Sf9 insect cells (unpublished results).

The function of GPCR palmitoylation, or at least the need for palmitoylation for proper folding and function in some GPCRs, is still controversial. Recently, we found that removal of palmitoylation from rhodopsin produced minor but cumulative defects in vision, mainly because of palmitoylation's stabilizing effect on the unliganded receptor (opsin).³⁸ On the other hand, palmitoyl chains have markedly different conformations in the various rhodopsin crystal structures determined to date, and the β_1 adrenergic receptor has been crystallized with the C358A mutation, removing the palmitoylation site altogether.³⁹ These recent results suggest that palmitoylation is not essential for crystallization of naturally palmitoylated GPCRs, but we have yet to learn what the effect(s) of heterogeneous palmitoylation, as in the case of 5-HT₄R expressed in mouse rod cells, would be on GPCR crystallization.

In summary, we have shown that heterogeneity of PTMs in membrane proteins can easily be overlooked by the SDS–PAGE and SEC metrics normally used to assess such constructs, potentially leading to negative effects on function–structure studies. In the case of 5-HT₄R, glycosylation, phosphorylation, and palmitoylation were heterogeneous with respect to both the identities and the total numbers of modified residues. This encouraged us to make changes in the construct and/or purification protocol to eliminate or at least minimize such heterogeneity prior to crystallization trials. Heterogeneity of glycosylation in rod cells appears to be receptor-dependent (or receptor family-dependent) because several GPCRs heterologously expressed in either *Xenopus* or mouse rod cells showed considerably less heterogeneity of glycosylation than 5-HT₄, 5-HT_{2A}, and 5-HT₇ receptors, as judged by electrophoretic criteria (this work, refs 9 and 11, and unpublished data).

■ ASSOCIATED CONTENT

● Supporting Information

A SEC profile, mass spectroscopy coverage and mass spectra for purified 5-HT₄R from TG mice, and SDS–PAGE gels and immunoblots for several GPCRs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

5-HT₄R, serotonin receptor type 4; AA₁R, adenosine subtype A1 receptor; DDM, *n*-dodecyl β -D-maltoside; GlcNAc, N-acetylglucosamine; GPCR, G-protein-coupled receptor; LC, liquid chromatography; Man, mannose; MS, mass spectroscopy; PTM, post-translational modification; ROS, rod outer segment(s); SEC, size-exclusion chromatography; TG, transgenic.

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