GPCRs in the Sweet Spot: Glycosylation and other PTMs

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Abstract

Post translational modifications (PTMs) are a fundamental phenomenon across all classes of life and several hundred different types have been identified. PTMs contribute widely to the biological functions of proteins and greatly increase their diversity. One important class of proteins regulated by PTMs, is the cell surface expressed G protein-coupled receptors (GPCRs). While most PTMs have been shown to exert distinct biological functions, we are only beginning to approach the complexity that the potential interplay between different PTMs may have on biological functions and their regulation. Importantly, PTMs and their potential interplay represent an appealing mechanism for cell and tissue specific regulation of GPCR function and may partially contribute to functional selectivity of some GPCRs. In this review we highlight examples of PTMs located in GPCR extracellular domains, with special focus on glycosylation and the potential interplay with other close-by PTMs such as tyrosine sulfation, proteolytic cleavage and phosphorylation.

Keywords: Glycosylation, tyrosine sulfation, proteolytic cleavage, G protein-coupled receptor, PTM interplay.

Major Types of GPCR Glycosylation and the Impact on Signaling

N-glycosylation

Glycosylation is among the most prevalent and complex protein PTMs, involving more than 200 known glycosyltransferases and a similar number of related enzymes and transporters known in man. Glycosylation can be subdivided into several different types, usually grouped by the glycosidic linkage involved. One of the most abundant and well-described types of glycosylation is N-linked glycosylation, which occurs at the consensus motif Asn–X–Ser/Thr (X ≠ Pro) with about 70% occupancy 1. Apart from a few exceptions, all GPCRs carry at least one putative N-glycosylation site in their N-terminal ectodomain or extracellular loops (ECLs). There is
considerable amount of literature describing effects of N-glycosylation on receptor trafficking, ligand responsiveness and down-stream signaling (Figure 1). However, the results have been quite variable, making it difficult to determine patterns that would define general functional roles for GPCR N-glycans.

The most consistent functional role of GPCR N-glycosylation relates to receptor folding and trafficking. The N-glycosylation occurs co-translationally in the endoplasmic reticulum (ER) and is therefore intimately involved in protein folding. The core-N-glycan composed of three glucose, nine mannoses and two N-acetylglucosamines (Glc3Man9GlcNAc2) is added by a multiprotein complex oligosaccharyltransferase (OST) on N-glycan acceptor sites \(^2\). The trimming of the N-glycan by glucosidases then allows calnexin and calreticulin to bind transiently to the glycan structure. These ER-resident lectin chaperones assist in protein folding and have an important role in protein quality control \(^2\). The role of GPCR N-glycans in folding is in general implicated indirectly by the observation that most receptors require at least one N-glycan for efficient transport to the cell surface. Examples include receptors from different subfamilies of GPCRs (rhodopsin-like, secretin-like and glutamate receptor families) [e.g. \(^3-14\)]. Many of these accumulate intracellularly without N-glycosylation, distinctively in the ER [e.g. \(^7, 8, 10, 14-16\)]. The precise mechanisms behind the impaired cell surface expression have, however, remained largely uncharacterized. One exception is the δ opioid receptor that was shown to interact with calnexin shortly after synthesis \(^9, 17\). Without this N-glycan-mediated interaction, a large fraction of immature receptors is targeted for ER-associated degradation. In addition, the non-glycosylated Cys27 variant was found to exit the ER prematurely, leading to transport of ligand-binding incompetent receptors to the cell surface \(^9, 17\). Although the majority of studies support the notion that N-glycosylation of GPCRs is important for folding and ER exit, there are also exceptions, such as M2 muscarinic acetylcholine \(^18\) and D1 dopamine \(^6\) receptors.

The role of N-glycosylation in GPCR signaling is less clear. In many studies, the loss of N-glycans was not found to alter receptor function [e.g. \(^11, 12, 18-20\)], yet others have reported impaired ligand binding or down-stream signaling [e.g. \(^5, 10, 21, 22\)]. Many of these latter studies, however, lack quantitative data on receptor cell surface expression, thus failing to distinguish the distinct effects of N-glycosylation on receptor expression and function. The lack of N-glycosylation may indirectly affect signaling output by decreasing the number of functional receptors transported to the cell surface, or alternatively, by enhancing internalization and downregulation of plasma membrane receptors. The latter possibility is supported by the observation that mature cell surface expressed κ
and δ opioid receptors show increased constitutive turnover without their N-terminal N-glycosylation, suggesting a stabilizing role for N-glycans at the cell surface. Consistently, non-glycosylated mutant neurokinin 1, D2 dopamine and κ and δ opioid receptors undergo enhanced ligand-mediated internalization when compared with the respective wild-type receptors.

Another explanation for the variable and unpredictable results of the effects of N-glycosylation on GPCR ligand binding and signaling is the divergent techniques that have been used to eliminate receptor N-glycosylation. The applied methods, such as glycosylation inhibitors, enzymatic deglycosylation and site-directed mutagenesis, each have advantages and disadvantages that may reflect in the outcome of functional assays. The use of the glycosylation inhibitor tunicamycin in cultured cells causes global inhibition of protein N-glycosylation and may not result in completely non-glycosylated receptors, which is also the case with peptide-N-glycosidase F-mediated deglycosylation. Neither of these two methods is applicable for the detailed analysis of individual glycosylation sites, but the latter method has the advantage that it can be used to specifically target mature cell surface receptors. Karpa et al. observed that enzymatically deglycosylated D5 dopamine receptors are able to bind the ligand [3H]SCH23390 in a normal manner, whereas receptors in tunicamycin-treated cells show more than 90% decrease in saturable ligand binding. Comparable and seemingly contradictory results were obtained for the luteinizing and follicle-stimulating hormone receptors when glycosylation inhibitors, deglycosylation or mutagenesis were used to disrupt receptor N-glycosylation. Altogether, these observations support the notion that N-glycosylation is not required for ligand binding once these individual GPCRs have reached the plasma membrane but have an indirect effect by controlling receptor folding or trafficking during biosynthesis.

The most frequently used method to disrupt N-glycosylation, mutation of the consensus sites, has provided valuable information on the functional role of individual sites. However, even this approach has some drawbacks. For example, disruption of the relaxin receptor glycosylation at position 303 by replacing Asn303 with Glu or Ser305 with Ala was found to result in reduced cyclic AMP production in cells treated with H2 relaxin. A significant reduction in potency was seen for both mutants, but the maximum response was found to be significantly reduced only for the former, reflecting differences in cell surface expression caused by the amino acid exchange rather than the loss of glycosylation. In the case of the protease-activated receptor-1 (PAR1), mutation of the two N-glycosylation sites in ECL2 at Asn250 and Asn259 to Glu results in reduced receptor cell
surface expression but exchanging the same amino acids to Ala has no effect on trafficking but rather enhance G-protein coupling efficiency.

More carefully controlled recent studies have investigated the functional role of N-glycosylation for mature plasma membrane receptors and have also assessed site-specific roles of individual N-glycosylation sites. In addition, new analysis methods have allowed direct measuring of the effect of N-glycans on ligand binding affinity. Using a quantitative fluorescence polarization/anisotropy peptide-binding assay and the purified calcitonin receptor N-terminal domain, Lee et al. discovered that a distinct N-glycan at Asn130 enhances ligand binding affinity about 10-fold. A single GlcNAc residue was sufficient for this effect. Similar changes in ligand binding were obtained by mutating Asn130 to Asp in the full-length receptor. Subsequently, the N-glycan at Asn130 was found to modulate N-terminal domain dynamics of the receptor by increasing the ligand on-rate and decreasing the off-rate. The PAR-1 N-glycans were also found to have distinct functional roles. Whereas N-glycans in the receptor N-terminal domain were found to control receptor trafficking and regulate protease sensitivity and specificity, the ECL2 N-glycans control G protein coupling specificity in response to receptor stimulation and thus take part in regulating signaling bias. Intriguingly, the receptor devoid of ECL2 N-glycans was found to be more effective at stimulating Gq-mediated phosphoinositide signaling, whereas the glycosylated wild-type PAR1 shows a greater efficacy at Gi12/13-dependent RhoA activation. The GPCR Smoothened (Smo) that is involved in the evolutionarily conserved Hedgehog pathway was also found to carry N-glycans that regulate signaling bias, as their absence results in a clear change in the ability of the mouse Smo to induce non-canonical signaling via Gαi3, although the ability to induce a G protein-independent signaling is unaffected. The study also reported a marked difference between the Drosophila and mouse Smo, as N-glycans of the former were found to be indispensable for folding and trafficking unlike was the case with the mouse receptor. Based on these findings, the authors hypothesized that the role of Smo N-glycosylation has evolved coincidently with the emergence of non-canonical signaling, with a role in protein folding and trafficking in flies and a novel role in signaling in vertebrates.

The final processing of N-glycans takes place in the Golgi with a substantial diversity in the terminal sugar residues. However, most reports on GPCRs so far have utilized techniques that remove intact N-glycans. Therefore, we still have limited understanding of the distinct N-glycan structures that may affect GPCR function and signaling. Recent studies have provided new information on the functional role of the terminal sugar structures. For the chemokine receptor
CCR7, which binds the two endogenous chemokines CCL19 and CCL21 differently \(^4\) (Figure 2), the sialylated N-glycans were shown to negatively affect receptor sensitivity to chemokines, leading to reduced T cell migration \(^5\), and it was postulated that the N-glycans regulate the access and dissociation of chemokines to the binding pocket. In another recent publication, it was demonstrated that the meningococcus pathogen, which causes meningitis in humans, utilizes N-glycans for GPCR allosteric activation. This was found to occur via interaction of the pathogen with the two sialylated N-glycans of the \(\beta_2\) adrenergic receptor (\(\beta_2\)AR) N-terminus, resulting in selective \(\beta\)-arrestin signaling \(^6\). Intriguingly, the species specificity of the meningococcus pathogen is based on a structural difference of sialic acids in humans versus other mammals, given another example of evolution of the functional role of GPCR N-glycans.

O-glycosylation

O-glycosylation (also called mucin-type O-glycosylation) occurs in the Golgi after protein folding and is initiated by the transfer of N-acetylgalactosamine (GalNAc) to the hydroxyl of a serine, threonine or in rare cases tyrosine residues \(^7\). The initiation step is facilitated by a family of up to 20 different GalNAc transferases (GalNAc-Ts), encoded by the differentially expressed polypeptide N-acetylgalactosaminyl transferase (GALNT) genes, which have overlapping yet distinct specificities \(^8\). This is in contrast to other types of glycosylation such as N-glycosylation, for which a single protein complex (OST) is facilitating the initial transfer of the glycan to the protein backbone. Consequently, individual O-glycosylation sites (and down-stream effects) can be tightly controlled in a temporal and spatial manner, depending on the panel of GalNAc-Ts expressed in a given cell or tissue \(^9\) (Figure 1B). Mucins are classical examples of O-glycoproteins. They are huge and highly O-glycosylated proteins often lining the epithelial surfaces of the body. However, most proteins going through the secretory pathway are predicted to carry single sites or small clusters of O-glycosylation \(^10\). Elongation of the initial GalNAc occurs in a stepwise manner so that monosaccharides are attached to the growing oligosaccharide chain by individual glycosyltransferases. O-glycans can vary in size and structure but are usually capped with terminal negatively charged sialic acids.

Recent development within genetic engineering and mass spectrometry sensitivity have led to an enormous expansion in the number of known O-glycosylation sites and to new discoveries of site-specific control of non-GPCR cell surface receptor function, such as the low-density lipoprotein
receptors LDLR and LRP2. There are now more than 60 GPCRs with detected O-glycosylation sites and over 350 GPCRs with predicted sites. However, the functional impact on downstream signaling and function of the vast majority of these newly identified GPCR O-glycosylation sites remains unexplored. Yet, in a handful of more focused studies the effect of GPCR O-glycosylation has been addressed. Early work on the prostaglandin E2 and D2 receptors suggest that O-glycans affect ligand binding affinity to these receptors. No exact sites were mapped, but several of the prostaglandin receptors have clusters of predicted O-glycans in their N-termini (predicted by the “NetOGlyc 4.0” algorithm), which likely are responsible for the observed effects. We have shown that N-terminal O-glycosylation modulates constitutive turnover of the δ-opioid receptor so that the lack of O-glycans leads to a decreased level of functional receptors at the cell surface. GPR15 and the V2 vasopressin and κ opioid receptors have also been shown to undergo O-glycosylation, although no functions were identified. More recently, red and green opsins across several vertebrate species and the mouse Smo were found to be O-glycosylated as well. Other examples include β1AR and the chemokine receptors CCR5 and CCR7. However, as discussed below, these receptors carry multiple PTMs in their N-termini, which can affect each other and should be considered collectively.

Other PTMs and potential crosstalk with O-glycosylation

Tyrosine sulfation

Tyrosine sulfation is an underexplored, but increasingly recognized PTM, which may affect the signaling of several classes of GPCRs. The sulfation of tyrosines is directed by two protein-tyrosine sulfotransferases (TPST1/TPST2) with different specificities. The modification takes place in the Golgi coinciding with O-glycosylation. Tyrosine sulfation has been reported for many different protein classes, including GPCRs, peptide hormones, blood clotting factors and adhesion molecules. Yet, estimates put the total number of tyrosine sulfation sites at 1% of all extracellular tyrosine residues, suggesting that we have only scratched the surface. The CC and CXC chemokine receptors generally carry tyrosine sulfation in their N-termini, which are important for ligand binding and downstream signaling. They usually contain multiple potential tyrosine sulfation sites in their extracellular N-termini, and several of these have been experimentally identified. Tyrosine sulfation of CKRs has been shown to enhance affinity of chemokines. However, sulfation of distinct tyrosine sites in a receptor can result in strikingly different effects for individual chemokines, illustrating that tyrosine sulfation can differentially fine-tune and regulate biased
signaling. As recently described, the chemokine receptors also have a striking pattern of potential O-glycosylation in their N-termini and the affinity of chemokine binding to CCR5 and downstream signaling is affected by both sialylation of N-terminal O-glycans and tyrosine sulfation. Likewise, polysialylation of CCR7 was reported to be important for the binding of CC chemokine ligand 21 (CCL21), but not for CCL19, and for dendritic cell trafficking, and the nearby tyrosine sulfation also affects ligand binding. The interplay between these two PTMs still needs to be explored in more detail, but it is conceivable that patterns of specific-glycosylation sites, glycan structures and tyrosine sulfation sites differentially affect chemokine binding, thereby providing a complex added layer of regulation. The CXC chemokine receptors seem to have a higher presence of N-glycosylation motifs and fewer predicted O-glycosylation sites, which may reflect differences in the regulation of this system, further adding to the complexity. The C5a and C3a chemokine receptors also carry tyrosine sulfation important for ligand binding and signaling. In contrast to most other PTM sites described here, except perhaps for N-glycosylation, the tyrosine sulfation sites in C3aR are located in the large ECL2, which interestingly also carries O-glycosylation sites. Virally encoded GPCRs also carry tyrosine sulfation and it has been shown that both the autocrine activation of the viral GPCR ORF74 from Kaposi sarcoma-associated herpesvirus (KSHV) and the chemokine binding by the human cytomegalovirus-encoded viral GPCR US28 are dependent on tyrosine sulfation in the N-termini. Moreover, O-glycosylation has also been found in the latter. The sphingosine-1-phosphate receptor represents a fascinating case, as tyrosine sulfation and O-glycosylation have been detected on the same residue (Tyr22). The sulfation is needed for proper receptor signaling in lymphocytes, whereas the role of the O-glycan(s) is unknown.

Proteolytic cleavage

Proteolytic cleavage is a widespread, irreversible PTM occurring across all classes of biological pathways. Proteolytic cleavage is not merely unspecific degradation, but can occur at specific positions facilitated by the more than 500 proteases in the human proteome, of which approximately 300 are identified to pass through the secretory pathway. The co-regulation of proteolytic cleavage by glycosylation is a well-described phenomenon for many proteins such as fibroblast growth factor 23 (FGF23), Tumor necrosis factor α and LDLR. Much less is known about GPCRs, but an increasing number of GPCRs across subfamilies is reported to undergo proteolytic cleavage of their N-termini, and several have detected or predicted glycosylation sites in
close vicinity, which could potentially affect the cleavage. The adhesion GPCRs with their exceptionally large N-terminal domains, are known to undergo autocatalytic cleavage at the conserved GPCR proteolysis site (GPS) located in the B-subdomain of the GPCR autoproteolysis-inducing (GAIN) domain. The cleaved, N-terminal fragment remains non-covalently associated with the C-terminal membrane-bound domains. Importantly, the adhesion GPCRs do not only undergo GPS cleavage, but several also undergo protease-mediated cleavage in their N-terminal domain, such as the orphan receptors GPR124 and BAI1 and BAI2 (Brain-specific angiogenesis inhibitors 1 and 2, respectively). The GPS cleavage takes place in the ER and can be affected by N-glycosylation as reported for CD97 antigen. Similarly, the membrane association of the N-terminal fragment of EMR2, resulting from cleavage, was shown to be dependent on site-specific N-glycosylation.

Among rhodopsin-type GPCRs, PARs are the most thoroughly characterized receptors, the function and signaling of which is regulated by proteolytic cleavage. All four receptors (PAR1-4) have either predicted or detected O-glycosylation sites in close proximity to the cleavage sites. Several GPCRs are also cleaved in an agonist-dependent manner, including β1AR and we and others have shown that O-glycosylation of β1AR can affect its downstream signaling by blocking the cleavage, which in turn controls signaling and may also induce signaling bias (Figure 2). Interestingly, the β1AR N-terminus also harbors a common polymorphic site at one of the O-glycosylated residues (position 49). The less common glycine variant cannot be O-glycosylated and shows a higher degree of desensitization and agonist promoted down-regulation compared to the more common serine variant. We found that the N-terminal O-glycosylation of β1AR is controlled by a single GalNAC-T isoform (GalNAC-T2), which was confirmed in the hearts of GalNAC-T2 knockout rats. However, β1AR has several glycosylation and cleavage sites and is expressed in many different tissues, thus it cannot be ruled out that the regulation is tissue-specific, highlighting the complexity added by the multiple PTMs and their interplay.

Phosphorylation and other PTMs

The most studied form of PTM for GPCRs is phosphorylation that is catalyzed in their intracellular domains by GPCR kinases (GRKs) and other kinases, including many second messenger kinases such as PKA, PKC, CaM kinases etc. Phosphorylation can be differentially regulated in a ligand- and tissue-dependent manner and has diverse roles in regulating receptor trafficking, signalling and regulation. The importance of GPCR phosphorylation has been reviewed extensively elsewhere.
and it is discussed in this review in the context of the potential for intersection of sites of phosphorylation with other PTMs. Many cytosolic and nuclear proteins carry O-GlcNAc glycosylation on specific serine or threonines and this is thought to occur in a “ying yang” relationship with phosphorylation by competing for the same or close-by acceptor sites. This dynamic interplay has only been reported for a limited number of plasma membrane proteins and it is unclear if the intracellular regions of GPCRs can also carry O-GlcNAc modifications. Interestingly, phosphorylation on serines, threonine and tyrosines has also been reported on extracellular domains on proteins that are transported through the secretory pathway. As reviewed elsewhere, many studies probing intracellular phosphorylation by mass spectrometry also detect phosphorylation sites in extracellular domains, but these are usually ignored. Phosphorylation in extracellular domains are thus underestimated and may show more general overlap and cross-talk with other PTMs, such as O-glycosylation and proteolytic cleavage. This has been extensively studied for the secreted FGF23, the function of which is regulated by cross-talk between O-glycosylation, phosphorylation and furin cleavage occurring within a few amino acids. Several transmembrane receptors such as the sortilin-related receptor and the transferrin receptor also carry phosphorylation sites in extracellular regions in close vicinity to O-glycosylation and proteolytic cleavage. The investigation of extracellular phosphorylation sites is however still in its infancy and to our knowledge no reports of sites in GPCRs have been published so far. However if the occurrence is as widespread as suggested, this may very well just be a matter of time.

Novel methods and possibilities have emerged

As mentioned above, there has been significant advancement in the knowledge of O-glycosylation sites in the human proteome and this has also improved prediction algorithms for unknown O-glycosylation sites. Similarly, mass spectrometry based technologies such as Terminal Amine Isotopic Labeling of Substrates (TAILS) has led to an expansion in known cleavage sites. Although methods have been developed, we have not yet seen the same expansion in the known sulfoproteome. It is evident that many GPCRs carry potential O-glycosylation sites, and this potential should be considered when probing ligand binding, GPCR function and downstream signaling, especially when comparing different cell lines and tissues, which likely have different glycosylation capacities. Genetic engineering has provided new tools to accurately dissect O-glycosylation, as well as N-glycosylation, in cell lines systems and this technology can be applied to other systems as well. However, removing specific transferases or proteases may affect
other PTM systems indirectly, as we have shown for GalNAc-T2. In this case, knocking out just one GalNAc-T caused global changes in the protease shield \(^9^1\). Similarly, TPSTs are O-glycosylated and glycosyltransferases undergo proteolytic cleavage. Therefore, complementary approaches for validation of the functional impact of individual PTM sites on specific receptors are needed.

**Future directions and perspectives**

To clearly grasp the regulation of GPCR signaling and ligand binding, we must consider PTMs as important players. Phosphorylation and disulfide bridges are well-established PTMs that are crucial for GPCR signaling and can also control biased signaling, as shown by us and others for instance in the chemokine system \(^9^2-^9^4\). In comparison, we have only begun to appreciate the multitude of PTMs on GPCR extracellular domains and even less so their potential interplay. There are still many outstanding fundamental biological questions. How are individual acceptor sites regulated? Can different enzymes e.g. TPSTs and GalNAc-Ts compete for the same residues in the secretory pathway? Can PTMs on GPCRs be remodelled at the plasma membrane or after internalization? To what extent do these PTMs co-regulate receptor functions through ligand binding, trafficking, stability or other mechanisms? And what about temporal and spatial considerations of these phenomena? We do not understand how widespread these PTMs are across the GPCR superfamily, but a substantial amount of information has emerged. With new technologies, we can now start to dissect these systems and their biological roles.

Figure 1: A) Several different PTMs can occur in the extracellular domains of GPCRs (most commonly in the N-terminus, but also in ECLs). N-glycosylation, O-glycosylation, tyrosine sulfation and proteolytic cleavage are among these PTMs and have been reported to be able to affect each other (shown by red and green arrows). GPCR functions, which can be affected by these PTMs, include ligand binding, signaling, internalization and trafficking. B) Glycosylation sites and structures are dependent on panel of glycosyltransferases in individual cells or tissues, and the same receptor therefore exists in multiple glycoforms. Here it is exemplified with O-glycosylation, but N-glycosylation, tyrosine sulfation and proteolytic cleavage can also vary depending expression patterns. C) Multiple enzymes control the initiation event. Two tyrosylprotein sulfotransferase (TPSTs) control tyrosine sulfation. Up to twenty Polypeptide N-acetylgalactosaminyltransferase (GalNAc-Ts) control O-glycosylation. One oligosaccharyltransferase complex, controls N-glycosylation initiation and approximately 300 proteases localized in either the secretory pathway.
or in the plasma membrane has been identified. Furthermore, both O- and N-glycosylation can exist in multimple glycoforms, leading to further complexity and regulatory potential.

Figure 2: Examples of PTM crosstalk in GPCRs. A, The primary sequence of the entire extracellular N-terminus of β1AR is shown with known sites of N-glycosylation, O-glycosylation and proteolytic cleavage. Functional effects are shown with red (inhibitory) or green (activating) arrows. O-glycosylation of β1AR has been shown to affect proteolytic cleavage, which in turn affects receptor signaling, turnover and internalization. Furthermore, a common SNP, leading to a serine to glycine substitution at position 49 (indicated with *), has been reported to result in lowered receptor signaling, potentially due to loss of O-glycosylation and stability. B, The primary sequence of the entire extracellular N-terminus of the chemokine receptor CCR7 with known PTMs is shown. Polysialic acid has been reported for CCR7 on both N- and O-glycans, but it is not clear which glycans on which acceptor sites are responsible for the reported effect on CCL19 binding. Here, polysialylation is shown on the N-glycan in the N-terminus for simplicity. Similarly, the inhibitory effect of N-glycan sialylation has been reported for Asn36 shown in the sequence here as well as for Asn292 in ECL3.

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Multiple PTMs in GPCR N-termini can affect receptor function and downstream signaling.
Examples of GPCRs with multiple PTMs

A

N-glycosylation facilitates surface expression?

O-glycosylation blocks cleavage

Cleavage attenuates signaling

B

Ligand binding

Polysialylation?

Ligand binding

CCL21 but not CCL19 binding

Polysialylation?
Post translational modifications (PTMs) contribute widely to the biological functions of proteins and greatly increase their diversity. PTMs and their potential interplay represent an appealing mechanism for cell and tissue specific regulation of GPCR function and may partially contribute to functional selectivity and regulatory control.