Palmitoylation of serotonin receptors

Nataliya Gorinski and Evgeni Ponimaskin
Cellular Neurophysiology, Hannover Medical School, Carl-Neuberg-Strasse 1, Hannover 30625, Germany

Abstract
The covalent attachment of palmitic acid to one or more cysteine residues (S-palmitoylation) is a widespread modification of signalling proteins. With the finding that palmitoylation is a dynamic process, it is now widely accepted that repeated cycles of palmitoylation/depalmitoylation could be involved in the regulation of multiple signalling processes. Palmitoylation also represents a common post-translational modification of the GPCRs (G-protein-coupled receptors). Functionally, palmitoylation of GPCRs has been shown to play a central role in the regulation of multiple receptor functions, including determining the efficiency and selectivity of G-protein coupling, receptor phosphorylation and desensitization, endocytosis and transport to the plasma membrane. The present review summarizes our current knowledge of the palmitoylation of serotonin (5-hydroxytryptamine) receptors and its role in the regulation of receptor functions.

Introduction
Cellular as well as viral proteins often undergo post-translational modifications. One of these modifications is a covalent attachment of long-chain saturated fatty acids (i.e. palmitate) to cysteine residue(s) within the protein via a labile thioester linkage (palmitoylation). Among the cellular palmitoylated proteins, polypeptides involved in signal transduction [e.g. receptors, G-protein α-subunits and ACs (adenylate cyclases)] are prevalent. With the finding that palmitoylation states of several proteins may be dynamically regulated, it is now widely accepted that repeated cycles of palmitoylation and depalmitoylation could have important functional consequences for signalling [1,2].

Palmitoylation represents a widespread post-translational modification of the GPCRs (G-protein-coupled receptors) [3]. These integral membrane proteins belong to the largest and most versatile group of receptors with an essential role in the regulation of almost all physiological processes in both mammalian and non-mammalian species, thus representing key pharmacological targets [4]. Palmitoylation of GPCRs seems to be a general feature of these signalling molecules since approximately 80% of all known receptors contain the potentially palmitoylatable cysteine residue(s) downstream of their seventh transmembrane domain [5]. For many GPCRs, palmitoylation has also been confirmed experimentally. The functions of GPCR palmitoylation cover the wide spectrum of their biological activities: from coupling to G-proteins and regulation of endocytosis to receptor phosphorylation and desensitization [1,6,7]. For example, studies on rhodopsin indicate the importance of its palmitoylation for the light-dependent GTPase activity of Gt and the light-independent activity of opsin–atr (all-trans-retinal) [8]. The functional characterization of non-palmitoylated β2-adrenergic and ETb (endothelin-B) receptors has revealed that palmitoylation is essential for agonist-stimulated coupling to Gt and to both Gt- and Gt-proteins respectively [9–11]. Analysis of the non-palmitoylated ETA (endothelin-A) receptor mutant demonstrated that ligand-induced stimulation of Gt was unaffected by the lack of palmitoylation, whereas signalling through Gt was prevented [12]. A later study on CCR5 (CC chemokine receptor 5) and prostacyclin receptors has also demonstrated that receptor palmitoylation is involved in the activation of their intracellular signalling pathways [13]. These findings show that receptor palmitoylation plays differing functional roles at different receptor–G-protein interfaces, suggesting that there is no common function applicable to all GPCRs. Therefore an analysis of the functions of palmitoylation is necessary for each individual receptor in order to understand its signalling mechanism.

The present review summarizes our current knowledge of the palmitoylation of serotonin receptors and its role in the regulation of receptor functions. Serotonin [5-HT (5-hydroxytryptamine)] is an important neurotransmitter and a local hormone that acts in the central nervous system as well as in various peripheral organs by activating a large family of receptors. On the basis of their structural and functional characteristics, serotonin receptors are divided into seven distinct classes [14]. With the exception of the 5-HT3 receptor, which is a transmitter-gated Na+/K+ -ATP channel, all other 5-HT receptors are members of the seven-transmembrane GPCRs. Palmitoylation was experimentally demonstrated for four 5-HT receptors, including 5-HT1A, 5-HT1B, 5-HT4 and 5-HT7. In addition, the members of 5-HT2 and 5-HT3 receptor subfamilies possess potential palmitoylation sites within their C-terminus, although palmitoylation of these receptors was not experimentally confirmed.

Key words: G-protein-coupled receptor (GPCR), palmitoylation, serotonin (5-hydroxytryptamine), signal transduction.

Abbreviations used: AC, adenylate cyclase; ERK, extracellular-signal-regulated kinase; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; 5-HT, 5-hydroxytryptamine.

To whom correspondence should be addressed (e-mail ponimaskin.evgeni@mh-hannover.de).
Palmitoylation of 5-HT1 receptors

The 5-HT1A receptor is the most extensively characterized member of the serotonin receptor family. This receptor is expressed in many brain regions such as the raphe nuclei, hippocampus and cortex, as well as in the gastrointestinal tract and platelets [14,15]. The 5-HT1A receptor couples to a variety of effectors via pertussis toxin-sensitive heterotrimeric G-proteins of the Gi/o family, and receptor activation results in the inhibition of AC and a subsequent decrease in intracellular cAMP levels. Activation of the 5-HT1A receptor also leads to a Gβγ-mediated activation of a K+ current, inhibition of a Ca2+ current, stimulation of phospholipase C, as well as an activation of the mitogen-activated protein kinase ERK (extracellular-signal-regulated kinase) 2 [14,16]. In addition, 5-HT1A receptor was demonstrated to form homo-oligomers [17] as well as heterodimers with the 5-HT2 receptor, which can affect the receptor-mediated signalling [18]. With respect to its physiological functions, the 5-HT1A receptor is involved in a wide range of physiological and pathological processes including regulation of mood, sleep and body temperature, as well as respiratory and cardiovascular control [19–21]. Considerable interest in this receptor has been raised due to its involvement in regulation of depression and anxiety states [22].

We have shown previously that the 5-HT1A receptor is modified by the covalently attached palmitate at its C-terminal cysteine residues Cys417 and Cys452 [23]. We have also demonstrated that receptor palmitoylation is irreversible and insensitive to the agonist stimulation [23]. Such a stable and agonist-independent palmitoylation is still unusual for GPCRs, which generally undergo repeated cycles of palmitoylation–depalmitoylation (see below). Interestingly, the 5-HT1A receptor possesses a very short C-terminus that is composed of only 18 amino acids and this could be a possible reason for the absence of a specific motif required for the recognition by the depalmitoylation enzyme(s). Alternatively, the orientation of the palmitate group within the membrane together with the basic amino acids surrounding palmitoylated cysteine residues and thus stabilizing tight association of the C-terminus with the plasma membrane may render them inaccessible to the enzyme.

The functional role of 5-HT1A receptor palmitoylation has been addressed by the analysis of its acylation-deficient mutants. When palmitoylated cysteine residues were mutated, communication between receptors and Gαi-subunits was completely abolished. Moreover, non-palmitoylated mutants were no longer able to inhibit cAMP formation, indicating that palmitoylation of the 5-HT1A receptor is critical for the enabling of G1-protein coupling/effector signalling. The receptor-dependent activation of ERK was also affected by acylation-deficient mutants, which suggests the importance of receptor palmitoylation for signalling through the Gβγ-mediated pathway [23].

How can palmitoylation of the 5-HT1A receptor mediate communication between receptor and G1-protein? Principally there are two possibilities: first, palmitoylation may be required to provide the proper conformation needed either for the receptor/G-protein binding or receptor-mediated G-protein activation. Alternatively, palmitoylation may be essential for receptor trafficking and/or localization to the membrane subdomains, such as rafts. The latter possibility was confirmed experimentally by demonstrating that a significant fraction of the wild-type 5-HT1A receptor resides in membrane rafts, whereas the yield of the palmitoylation-deficient receptor in these microdomains is significantly reduced [24]. Since lipid rafts often work as a platform for co-localization of GPCRs with corresponding G-proteins, the palmitoylation-dependent raft localization of the 5-HT1A receptor appears to be critically involved in receptor-mediated signalling.

In addition to the functional analysis of 5-HT1A receptor palmitoylation, a possible relationship between receptor palmitoylation and oligomerization has been investigated in two previous studies [17,25]. Using a novel FRET ( Förster resonance energy transfer) technique based on spectral analysis, these authors demonstrated that FRET efficiency measured for the wild-type oligomers significantly decreased in response to agonist stimulation. The combined results of these studies suggest that this decrease was mediated by accumulation of FRET-negative complexes rather than by dissociation of oligomers to monomers. By contrast, the agonist-mediated decrease of FRET signal was completely abolished in oligomers composed by the non-palmitoylated receptor mutants, demonstrating the importance of palmitoylation in modulation of the structure of oligomers. These authors also suggested a molecular model to explain how palmitoylation may influence oligomerization and functions of the 5-HT1A receptor (Figure 1). In this model palmitoylation does not directly modulate oligomerization of 5-HT1A, but rather serves as a targeting signal responsible for the retention of the 5-HT1A receptor in defined membrane microdomains.

In addition to the 5-HT1A receptor, another member of the 5-HT1 receptor subfamily, the 5-HT1B receptor, has also been shown to be palmitoylated [26]. However, it still remains to be determined whether palmitoylation is involved in regulation of the function of the 5-HT1B receptor.

Palmitoylation of the 5-HT4 receptor

The 5-HT4 receptor is expressed in a wide variety of tissues including the brain, colon, urinary bladder, gastrointestinal tract and heart [27]. In the mammalian brain the 5-HT4 receptor contributes to the control of acetylcholine and dopamine secretion, facilitates cognitive performance and is also implicated in anxiety [28]. Furthermore, the 5-HT4 receptor is thought to be involved in various central and peripheral disorders, including neurodegenerative diseases, major depressive disorders and anorexia [29,30]. The wide distribution of 5-HT4 receptors is paralleled by the existence of many 5-HT4 splicing variants. Functional expression was reported for five C-terminal and one internal splice variant in humans [31]. In mice, four 5-HT4 receptor isoforms, 5-HT4a, 5-HT4b, 5-HT4c and 5-HT4d, have been cloned [32].
All of these variants, except for the internal splice product in humans, share the same sequence up to Leu358 followed by an unique C-terminus. Since the C-terminal tail seems to be involved in the fine-tuning of the coupling of GPCRs to G-proteins, it has been proposed that a diversity of function can be attributed to the different splicing variants of the 5-HT4 receptor [33,34].

The 5-HT4 receptors are positively coupled with AC through a G11-protein and receptor-mediated increase in cAMP levels leads to phosphorylation of a number of target proteins, including voltage-gated channels [35,36]. The heterotrimeric G11-protein represents an additional interaction partner of the 5-HT4 receptor. Activation of the 5-HT4 receptor/G11 pathway results in stimulation of RhoA GTPase leading to reorganization of actin cytoskeleton [37]. It has also been shown that the 5-HT4 receptor can activate the Src/ERK pathway in a G-protein-independent manner [38]. Thus the diversity of interaction partners represents an additional molecular basis for the complexity of 5-HT4 receptor signalling.

The mouse 5-HT4(a) receptor is covalently modified by palmitic acid. Receptor palmitoylation was demonstrated to be a reversible process and receptor stimulation increases the turnover rate for receptor-bound palmitate [39]. In a follow-up study, it was shown that the 5-HT4(a) receptor contains two potential palmitoylation sites (Cys328/Cys329 and Cys386) located in the C-terminal domain of the receptor [40]. Noteworthily, both of these cysteine residues are presented in all 5-HT4 receptor isoforms, suggesting their potential palmitoylation. The finding that the conserved cysteine residues Cys328/Cys329 are modified with palmitic acid is consistent with a general view of the location of palmitoylation sites on GPCRs. In contrast, the existence of an additional palmitoylated Cys386 positioned 70 amino acids away from the plasma membrane face and close to the C-terminal end of the 5-HT4(a) receptor was surprising. Thus the 5-HT4(a) receptor represents the first case of palmitoylated receptors with the novel acylation site located close to its C-terminus.

Functional analysis of palmitoylation-deficient mutants revealed that the coupling of non-palmitoylated receptors with the G11-protein agonist-promoted cAMP production as well as intracellular distribution was unaffected. However, palmitoylation was critically involved in the modulation of the receptor’s constitutive activity. Noteworthily, mutation of the proximal palmitoylation site (Cys328/Cys329) resulted in a significant increase in the receptor’s capacity to convert from the R (inactive) into the R* (active) form in the absence of an agonist. By contrast, the rate of isomerization from R to R* for the C386S mutant as well as for the triple non-palmitoylated mutant (C328S/C329S/C386S) was similar to that obtained for the wild-type. Generally, these data suggest that palmitoylation of the 5-HT4(a) receptor is directly involved in isomerization of the receptor.
Figure 2 | Hypothetical model for regulation of 5-HT$_4$ and 5-HT$_7$ receptor activity by dynamic palmitoylation

Depending on the number of palmitoylated cysteine residues, there are four receptor populations: those with two, one small, one large or no intracellular C-terminal loops. These populations exist in dynamic equilibrium, and every conformation could be changed to one of the remaining three forms by basal or agonist-promoted palmitate turnover. 5-HT$_4$R, 5-HT$_7$R, receptor, the receptor in a constitutively active conformation for $G_s$-mediated signalling.

from R to R$^*$ by dictating conformation of its flexible cytoplasmic loops involved either in the receptor/G-protein recognition process or in G-protein binding and/or receptor-mediated G-protein activation (Figure 2). Interestingly, the C328S/C329S mutant also exhibited enhanced receptor phosphorylation under both basal and agonist-stimulated conditions [41]. Moreover, this mutant was more effectively desensitized and internalized via a β-arrestin-mediated pathway compared with the wild-type receptor. In contrast, G-protein activation, phosphorylation, desensitization and internalization of the other palmitoylation-deficient receptor mutants were affected differently [41]. These findings further confirm functional significance of the 5-HT$_4$R receptor palmitoylation and suggest that G-protein activation, phosphorylation, desensitization and internalization depend on the conformation of intracellular C-terminal receptor domain regulated by palmitoylation. Since both the phosphorylation and the palmitoylation status of the 5-HT$_4$R receptor are dynamically regulated by agonist stimulation, concerted interaction of these two post-translational modifications seems to play an essential role in modulating the function of 5-HT$_4$R receptors.

Palmitoylation of the 5-HT$_7$ receptor

The 5-HT$_7$R receptor is one of the most recently described members of the 5-HT receptor family [42]. This receptor stimulates cAMP formation by activating AC via $G_s$-proteins [43]. In addition, the 5-HT$_7$R receptor is coupled to the $G_{12}$-protein to activate small GTPases of the Rho family, which leads to enhanced neurite outgrowth, synaptogenesis and neuronal excitability [44,45]. Three 5-HT$_7$R receptor isoforms [5-HT$_7$(a), 5-HT$_7$(b) and 5-HT$_7$(d)], differing in the amino acid sequence of their C-terminus and possessing a similar pharmacological profile, were cloned in rat and human tissues [46].

The 5-HT$_7$R receptor is associated with a number of physiological and pathophysiological responses, including serotonin-induced phase shifting of the circadian rhythm [47] and age-dependent changes of the circadian timing [48]. In addition, a large body of evidence indicates an involvement of the 5-HT$_7$R receptor in the development of anxiety and depression, and studies have shown that the 5-HT$_7$R receptor can be clinically relevant for the treatment of major depressive disorders [49].

The mouse 5-HT$_7$(a) receptor has been shown to undergo post-translational modification by palmitate, which is covalently attached to the protein through a thioester-type bond. Similarly to the 5-HT$_4$R receptor, the 5-HT$_7$(a) receptor is dynamically palmitoylated in an agonist-dependent manner, so that previously synthesized receptors may be subjected to repeated cycles of palmitoylation–depalmitoylation [50]. Mutation analysis revealed that Cys$^{424}$ and Cys$^{438}$/Cys$^{441}$ located in the C-terminal receptor domain represent the potential palmitoylation sites. It is also of note that, although Cys$^{424}$, Cys$^{438}$ and Cys$^{441}$ are responsible for the attachment of 90% of the receptor-bound palmitate, palmitoylation of the 5-HT$_7$(a) receptor is not restricted to its C-terminus. Further experimentation will therefore be necessary to identify additional cysteine residues involved in receptor palmitoylation.

Functional analysis of acylation-deficient mutants revealed that non-palmitoylated 5-HT$_7$(a) receptors were indistinguishable from the wild-type for their ability to interact with $G_s$- and $G_{12}$-proteins after agonist stimulation. In contrast, mutation of the proximal palmitoylation site C404S (either
alone or in combination with C438S/C441S significantly increased the agonist-independent G\textsubscript{\textalpha}s-mediated constitutive 5-HT\textsubscript{7(a)} receptor activity, whereas the activation of G\textsubscript{\textalpha}12-protein was not affected.

In combination with the previous findings on the functional role of 5-HT\textsubscript{4(a)} receptor palmitoylation, this suggests that palmitoylation can represent a general feature regulating constitutive receptor activity. In the case of the 5-HT\textsubscript{7(a)} receptor, which is coupled with both G\textsubscript{\textalpha}s and G\textsubscript{\textalpha}12-proteins, dynamic palmitoylation can also represent a molecular mechanism responsible for selective G\textsubscript{\textalpha}s- or G\textsubscript{\textalpha}12-mediated signalling. Such regulatory effects of 5-HT\textsubscript{7} palmitoylation may also be relevant in other species such as mouse and in particular in humans. Indeed, the proximal C-terminal cysteine residue is highly conserved among all 5-HT\textsubscript{7} receptor isoforms, including human splicing variants 5-HT\textsubscript{7(a)}, 5-HT\textsubscript{7(b)} and 5-HT\textsubscript{7(d)} [46]. In addition, the human receptor isoform 5-HT\textsubscript{7(a)} possesses a distal cysteine residue, Cys\textsuperscript{341}, which might represent an additional acylation site. Using data obtained for the 5-HT\textsubscript{4} receptor, one can also suggest that molecular mechanisms regulating the constitutive activity of 5-HT\textsubscript{7} and 5-HT\textsubscript{7} receptors may be quite similar (Figure 2). In both cases dynamic palmitoylation can be responsible for formation of additional flexible C-terminal intracellular loop(s) involved either in G-protein binding or receptor-mediated G-protein activation by providing a lipophilic membrane anchor. Such a mechanism, in combination with a dynamic receptor palmitoylation, can also explain the higher constitutive activity reported for the 5-HT\textsubscript{4(a)} and 5-HT\textsubscript{7(a)} receptors in neurons [32,33,51,52]. Indeed, dynamic receptor palmitoylation implies that at any given point, populations of differently palmitoylated receptors are present in the cell, also including the forms with a high constitutive activity (Figure 2).

Funding

This study was supported by the Fund of the Hannover Medical School and by the Deutsche Forschungsgemeinschaft [grant number PO 732].

References

10 O’Dowd, B.F., Hnatowich, M., Caron, M.G., Lefkowitz, R.J. and Bouvier, M. (1989) Palmitoylation of the human β2-adrenergic receptor. Mutation of Cys\textsuperscript{341} in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. J. Biol. Chem. 264, 7564-7569


34 Claeyssen, S., Sebben, M., Becamel, C., Eglen, R.M., Clark, R.D., Bockaert, J. and Dumuis, A. (2000) Pharmacological properties of 5-hydroxytryptamine(4) receptor antagonists on constitutively active 5-hydroxytryptamine(4) receptor splice variants. Mol. Pharmacol. 58, 136-144


Received 11 September 2012
doi:10.1042/BS12010235