Regulation of the orphan receptor Gpr176 activity via post-translational modifications in the central circadian clock(Abstract_要旨)

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論文題目	Regulation of the orphan receptor Gpr176 activity via post-translational modifications in the central circadian clock （概日時計中枢における翻訳後修飾を介したオーファン受容体Gpr176の活性調節）		

（論文内容の要旨）

G-protein-coupled receptors (GPCRs) are important drug targets with diverse therapeutic applications. However, there are still more than a hundred orphan GPCRs, whose protein functions and biochemical features remain unidentified. Gpr176 is one of these so-called orphan GPCRs. *Gpr176* (also known as *HB-954*) was initially cloned by Hata *et al.* from a human brain cDNA library. A previous study in our laboratory revealed that in the mouse brain, *Gpr176* is most abundantly expressed in the suprachiasmatic nucleus (SCN), the locus of the principal circadian pacemaker in the brain and demonstrated that this GPCR is required for keeping the pace of normal circadian behavioural activity rhythm, by generating and analysing *Gpr176*-deficient mice. Therefore, Gpr176 has been regarded a promising therapeutic target for insomnia and lifestyle-related diseases. However, biochemical characteristics are largely unknown for Gpr176. In this study, I focused on characterizing protein function and biochemical feature of Gpr176 and discovered that the level of Gpr176 activity is regulated by *N*-glycosylation (**Chapter 1**) as well as phosphorylation (**Chapter 2**).

Chapter 1: Identification and functional characterisation of *N*-linked glycosylation of the orphan G-protein-coupled receptor Gpr176.

N-glycosylation is one of the most common post-translational modifications for GPCRs. However, not surprisingly, knowledge regarding the presence and potential functional role of *N*-linked glycosylation for orphan GPCRs has been particularly sparse, compared to that of receptors with known ligands. Previous studies based on GPCRs with known ligands demonstrated that *N*-glycosylation may be important for their structural maturation, cell surface expression, ligand binding, and downstream signal transduction. However, the functional role(s) of *N*-glycosylation varied depending on the type of GPCRs tested. Because of this non-redundant functional nature of *N*-glycosylation, its role for Gpr176 must be determined empirically.

In this chapter, I first provide evidence that Gpr176 is *N*-glycosylated *in vivo*. Peptide-*N*-glycosidase treatment of mouse hypothalamus extracts revealed that endogenous Gpr176 undergoes *N*-glycosylation. Using a heterologous expression system, I then obtained evidence that *N*-glycosylation occurs at four conserved asparagine residues in the N-terminal region of mouse Gpr176 (N4, N11, N17 and N26). Prevention of *N*-glycosylation by the mutation of these sites led to a drastic reduction in Gpr176 protein expression. Non-glycosylated mutant proteins were mostly retained in the endoplasmic reticulum (ER) and readily degraded via the proteasomal proteolysis, suggesting a problem during protein synthesis in the ER. At the molecular function level, Gpr176 has constitutive, agonist-independent activity that leads to reduced cAMP synthesis. Although deficient *N*-glycosylation did not compromise this intrinsic activity, the resultant reduction of cell-membrane Gpr176 protein expression was accompanied by attenuation of cAMP-repressing activity in cells. My data therefore demonstrate that *N*-glycosylation is a prerequisite for the efficient protein expression of functional Gpr176.

Chapter 2: Identification of regulation of Gpr176 activity via phosphorylation by T-207219 and other non-orphan GPCRs.

Phosphorylation is a key regulatory post-translational modification described for GPCRs. In canonical

GPCR-mediated signaling pathway, agonist-activated receptors are phosphorylated in their cytoplasmic regions, resulting in subsequent receptor internalization and degradation. However, little is known about phosphorylation and its potential function(s) for orphan GPCRs. Because unlike other GPCRs, Gpr176 has a relatively large cytoplasmic C-terminal tail region composed of 181 amino acids, where several phosphorylatable serine/threonine residues are conserved, it is tempting to speculate that this region may be involved in the regulation of Gpr176 via phosphorylation.

In the 2nd chapter, I describe the identification and characterisation of phosphorylation that occurs on Gpr176. Using a high-throughput chemical library screening, I first identified T-207219 as a regulator for Gpr176, which induces phosphorylation at two separate conserved serine residues in the C-terminal region of Gpr176, causing receptor internalization and degradation. Moreover, using a receptor co-expression system, I obtained evidence that ligand-mediated activation of non-orphan GPCRs leads to phosphorylation of the same residues in Gpr176. Induced phosphorylation, importantly, caused reduced cell-surface expression and second messenger signaling of Gpr176. My data therefore identified a previously uncharacterised inter-GPCRs mechanism in which the orphan receptor is regulated by non-orphan receptors through phosphorylation.

Based on the results from **Chapters 1-2**, I have, for the first time, identified biochemical features of Gpr176 and discovered that the level of Gpr176 activity is regulated by post-translational modifications. My findings therefore provide an important basis for understanding GPCR regulation in the SCN and for developing Gpr176-targeted therapeutics.

（論文審査の結果の要旨）

Gタンパク質共役型受容体（GPCR）は、薬理学上最も重要でかつ効率の良いターゲットとして知られる分子群である。しかし、未だに100種類以上のオーファンGPCRが存在し、そのタンパク質機能や生化学的特徴が明らかにされていない。オーファン受容体Gpr176は、体内時計の最高位中枢器官である視交叉上核（SCN）に特異的に発現し、生体リズム調整能を有するオーファンGPCRであることから、生体リズム異常を伴う不眠症や生活習慣病に対する新しい治療薬の標的となることが期待される。しかしこれまでGpr176の活性調節につながる受容体タンパク質の生化学的特徴は明らかにされてこなかった。このような中、王　甜宇氏は、マウス視床下部SCNにおいてGpr176の翻訳後修飾の発見とその役割を明らかにした。第一章では、独自に作出した抗体を用いて、SCNに発現するGpr176のN末端領域が*N*型糖鎖修飾を受けることを見出した。この修飾はGpr176の効率的なタンパク質発現に必要であり、Gpr176 *N*型糖鎖修飾変異体は発現量低下に伴い細胞内での基礎活性レベルが低下することを証明した。第二章では、独自に構築した細胞アッセイ系を用いて化合物ライブラリーによる網羅的スクリーニングを行い、Gpr176の基礎活性調節分子として低分子量化合物T-207219を同定し、さらにその発見を契機に、本化合物作用に伴うGpr176のC末端領域に起こるリン酸化修飾とその後のリン酸化を介したGpr176の細胞内移行を明らかにした。このように、本論文は、Gpr176の生化学的特徴を特定し、Gpr176の細胞内活性レベルが*N*型糖鎖修飾とリン酸化という独立した2つの翻訳後修飾により巧妙に制御されることを明らかにした。これら一連の研究成果は、オーファン受容体の分子機能解明という創薬科学研究における重要な基盤を形成する所見を提供するとともに、脳内中枢時計における未解明のGタンパク質シグナル制御の実体理解に資するものである。したがって本研究はGpr176の新たな翻訳後制御の解明を通じて薬学・医学に貢献する重要な知見をもたらしたといえる。よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、２０２３年２月１６日に、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、（令和８年３月２３日までの間）当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日：　　年　　月　　日以降