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Characterization of ciliary targeting sequence of rat melanin-concentrating hormone receptor 1

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ABSTRACT

Melanin-concentrating hormone (MCH) is the natural peptide ligand for MCHR1 and MCHR2, which belong to the G protein-coupled receptor (GPCR) superfamily. The MCH-MCHR1 system is involved in the regulation of feeding, energy homeostasis and emotional processing in rodents. Recently, MCHR1 expression was discovered in neuronal immotile primary cilia of the central nervous system in mice. The cilium has an important chemosensory function in many types of cell and ciliary dysfunction is associated with cliopathies such as polycystic kidney disease, retinal dystrophy, and obesity. The targeting sequence of ciliary membrane proteins is thought to be unique. Although these sequences have been predicted in the cytoplasmic third loop and/or C-terminus of GPCRs, little is known about the characteristics of MCHR1. We thus explored the molecular mechanisms of MCHR1 targeting by transiently expressing a series of MCHR1 mutants into ciliated hRPE1 cells and evaluated the effects of these mutations on the ciliary localization of the heterologous receptor. This approach demonstrated that an Ala-to-Gly mutation (A242G) within the third intracellular loop induced a significant reduction in ciliary localization of the receptor without affecting the ciliogenesis. In contrast, no C-terminal truncation mutant had any effect on ciliary localization or cilia length. This study provides a potential molecular link between defective cilia and clinical manifestations such as obesity.

1. Introduction

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide first identified in the pituitary gland of salmon (Kawauchi et al., 1983). MCH is highly expressed in the lateral hypothalamus and zona incerta in rodents, both of which have extensive projections throughout the brain (Bittencourt et al., 1992). Acute intracerebroventricular injections of MCH transiently stimulates food intake in rats, while chronic infusion of MCH or MCH analogs significantly increases food intake, body weight, white adipose tissue mass and liver mass in mice fed a moderately high-fat diet *ad libitum* (Ito et al., 2003; Rossi et al., 1997). Further lines of evidence for the importance of MCH in feeding come from studies wherein the expression levels of MCH were manipulated by knockout and overexpression techniques (Alon and Friedman, 2006; Ludwig et al., 2001; Shimada et al., 1998). To our knowledge, as no other neuropeptides exhibit this phenotype, MCH seems to be a unique neuropeptide involved in feeding behavior. The effects of MCH are mediated through two G protein-coupled receptors (GPCRs), MCHR1 and MCHR2 (An et al., 2001; Chambers et al., 1999; Saito et al., 1999), both of which are widely expressed at high levels in the brain (An et al., 2001; Saito et al., 2001). Rodents possess only MCHR1, whereas other species including humans also express MCHR2 (Tan et al., 2002). The phenotypes of mice lacking MCHR1 are lean, hyperactive and hypermetabolic (Chen et al., 2002; Marsh et al., 2002) suggests that the pharmacological effects of MCH in rodents are solely mediated *via* MCHR1. In agreement with this, a peptidic MCHR1 antagonist that is without effect in the MCHR1 knockout mouse remarkably improved diet-induced obesity (DIO) in animals expressing the wild-type MCHR1 (Mashiko et al., 2005). Recently numerous small-molecule MCHR1 antagonists have been developed and consistently effective in rodent DIO (Luthin, 2007; Takekawa et al., 2002). Moreover, current evidence shows that MCHR1 antagonists are efficacious in animal models of depression, anxiety, sleep disturbance, and reward (Borowsky et al., 2002; Chung et al., 2011). Therefore, it appears that MCHR1 antagonists may have many potential applications in human diseases.

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The primary cilium is a ubiquitous organelle that projects from most vertebrate cells (Emmer et al., 2010; Louvi and Grove, 2011). Most of neurons in rodents have also possessed the primary cilia (Bishop et al., 2007). The role of primary cilia is to detect extracellular signals through a receptor or channel and transmit this diverse information to the cell body (Emmer et al., 2010; Louvi and Grove, 2011). The functions of cilia are defined by the signaling proteins expressed on their membranes, so specific targeting of these transmembrane receptors and channels is essential for ciliary function. Recent reports have shown that a subset of neuronal cilia express a specific inventories of GPCRs, including MCHR1, somatostatin receptor 3 (SSTR3) and serotonin receptor 6 ($5HT_6$) *in vivo* and *in vitro*, whereas somatostatin receptor 5 (SSTR5) and serotonin receptor 7 ($5HT_7$) are non-ciliary receptors (Berbari et al., 2008a,b; Miyoshi et al., 2009). Indeed, the physiological importance of neuronal cilia is illustrated by the gamut of diseases (ciliopathies) (Hildebrandt et al., 2011). For example, Bardet-Biedl syndrome (BBS) comprises several clinical manifestations such as obesity, diabetes mellitus, hypertension, retinopathy, renal defects, and polydactyly (Guo and Rahmouni, 2011). Notably, MCHR1 fails to localize to neuronal cilia in the olfactory tubercle, hypothalamus, and nucleus accumbens in a mouse BBS model characterized by obesity (Berbari et al., 2008a). Thus, proper targeting of MCHR1 to the cilia is indispensable for maintaining the cellular homeostasis in an incessantly changing extracellular environment and, in turn, controlling the body weight. In this context, extensive analysis of MCHR1 as a ciliary GPCR could provide a mechanistic link between ciliary defects and obesity.

Although the ciliary membrane is contiguous with the plasma membrane, ciliary localization of proteins is tightly regulated, with only certain GPCRs permitted to traffic there (Berbari et al., 2008a,b; Miyoshi et al., 2009). The molecular mechanism underlying this specific transportation of GPCRs to the cilia lies in a specific targeting sequence that enables interaction of GPCRs with an adaptor protein(s), making this complex to be trafficked to the cilia compartment (Emmer et al., 2010). By searching a database for all human GPCRs, consensus ciliary targeting sequences were predicted to be a conserved motif "Ax[S/A]xQ" or "AxxxQ" in the third intracellular (i3) loop and a "VxP/LxP" motif in the C-terminus (Berbari et al., 2008b; Domire et al., 2011). Within the C-terminal tail, the "FK/FR" motif of the juxtamembrane sequence of the seven-transmembrane protein, *smoothened*, is another putative ciliary localization signal (Corbit et al., 2005). A chimera of SSTR5 containing the i3 loop of SSTR3 (SSTR5-$SSTR3^{i3\ loop}$) localized to cilia when transfected into IMCD cells, but this phenomenon was blocked by mutation of the first and fifth amino acids of the i3 loop motif to Phe (Berbari et al., 2008b), indicating the role of the SSTR3 in ciliary localization. Conversely, replacing the i3 loop in SSTR3 with the i3 loop from SSTR5 (SSTR3-$SSTR5^{i3\ loop}$) did not prevent ciliary localization, indicating that the i3 loop alone is insufficient for this targeting. Another chimeric study using CD8α containing the i3 loop of SSTR3 also demonstrated ciliary trafficking, providing further evidence for the existence of a ciliary targeting sequence within the i3 loop motif (Jin et al., 2010). However, data from these chimeric approaches may confounded by the likely disruptive effects of these molecular biological modifications on GPCR conformation. To further characterize the ciliary targeting sequence in GPCRs, we expressed Flag-tagged point mutants of full-length rat MCHR1 in the ciliated human cell line, hRPE1. Screening from over 20 substitution mutants, we conformed that the mutation of third residue Ala to Gly (A242G) in the i3 loop motif significantly and selectively affected the ciliary localization of MCHR1, the result deepening our understanding of the molecular mechanism by which MCHR1 is targeted to the primary cilia.

2. Materials and methods

2.1. cDNA constructs for MCHR1 and mutant receptors

The generation of a cDNA encoding a Flag epitope tag before the first methionine in rat MCHR1 was described previously (Saito et al., 2003). Single-substitution mutations of Flag-MCHR1 were produced by oligonucleotide-mediated site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All mutations in the MCHR1 cDNA sequence were confirmed by sequencing analysis. Mutated MCHR1 cDNAs were excised by digestion with EcoRI and XhoI and inserted into the pcDNA3.1 expression vector.

2.2. Cell culture and transient transfection

hTERT-RPE1 epithelial cells (hRPE1) were grown in Dulbecco's modified Eagle's medium/F12 culture medium with 10% fetal bovine serum, 0.5 mM sodium pyruvate, 15 mM HEPES (pH 7.5), 10 μg/ml hygromycin B, and 1% penicillin/streptomycin, at 37 °C and 5% CO_2. Cells were seeded on 6-well plate at a density of 5×10^5 cells/well for 16–18 h. Plasmid DNA (2.0 μg) was mixed with LipofectAMINE 2000 transfection reagent (Life Technologies Corporation, Carlsbad, CA, USA), diluted with OptiMEM and added to cells. For immunocytochemistry, cells were re-plated onto Lab-Tech eight-well plates (Nunc, Rochester, NY, USA). To induce the growth of cilia in confluent cultures, the complete medium was replaced with serum-free medium 4 h after reseeding, after which cells were cultured for a further 18–20 h. The transfection efficiency of wild-type and mutant receptors was always between 25 and 30%.

2.3. Immunofluorescence microscopy

Transfected hRPE1 cells were fixed in a 3.7% paraformaldehyde–phosphate-buffered saline (PBS) solution for 10 min. After two washes with PBS, the cells were transferred into a blocking solution (20% goat serum in PBS) for 30 min, then incubated with polyclonal rabbit anti-Flag M2 antibody (1:500, Sigma) and monoclonal mouse anti-acetylated α-tubulin (AcTub, 1:250; Sigma) for 2 h. The 1st antibody was detected using the appropriate secondary antibodies (Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), respectively). The number of transfected cells and the number of these transfected cells with obvious ciliary localization of receptor were counted in each image (captured under a BX51 fluorescence microscope (Olympus, Tokyo, Japan)) by individuals blinded to the experimental conditions. The results are expressed as the percentage (mean ± SEM) of transfected cells exhibiting ciliary receptor localization. A total of 500 cells were counted from at least three independent transfections for each construct. Double-fluorescence imaging was also performed on the same cells using a FLUOVIEW FV1000 confocal microscope system (Olympus, Tokyo, Japan).

2.4. Molecular modeling

To generate a homology model of rat MCHR1, we used the X-ray structure of rhodopsin (PDB code 1U19), the classical structural template for GPCRs. Alignment analysis in sequences of rat MCHR1 and rhodopsin was performed using CLUSTALW2.0 installed in Genetyx v9.0 (Genetyx Corporation, Tokyo, Japan). An initial model of rat MCHR1 was constructed using the Modelor module installed in Discovery Studio v3.0 (Accelrys, Tokyo, Japan). The obtained