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Molecular cloning of a novel putative G protein-coupled receptor (GPCR21) which is expressed predominantly in mouse central nervous system

Yoshinaga Saeki*, Satoshi Ueno**, Ryuzo Mizuno, Tomoya Nishimura, Harutoshi Fujimura, Yoshitaka Nagai, Takehiko Yanagihara

Department of Neurology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan

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A novel cDNA clone encoding a putative G protein-coupled receptor (named GPCR21) was isolated from a mouse brain cDNA library along with its homologue, GPCR01 (the mouse counterpart of previously reported rat receptor R334 [(1991) FEBS Lett. 292, 243–248]) by the polymerase chain reaction using degenerate oligonucleotide primers. Northern blotting and reverse transcription-polymerase chain reaction analyses showed predominant expression of these two receptors in the central nervous system. In situ hybridization analysis revealed their prominent expression in the limbic system and further demonstrated the differential distribution of their mRNAs in mouse brain. Although the ligands for these receptors are yet to be identified, the significant sequence homology between these receptors suggests that they constitute a new receptor subfamily and they possibly represent different receptor subtypes for an unknown neurotransmitter.

G protein-coupled receptor; Polymerase chain reaction; Homology probing; Limbic system; Medial habenular nucleus; Testis; Mouse brain

1. INTRODUCTION

The wide spectrum of intercellular signals mediated by hormones, neurotransmitters and neuropeptides are transduced into cells via their specific receptors that interact with the guanine nucleotide-binding regulatory protein (G protein) signaling system [1,2]. Recent molecular cloning studies have led to the identification of a growing number of receptors of this class, G protein-coupled receptors [3,4,5]. Sequence comparison of these receptors suggests that all members of the receptor family have a specific tertiary structure in common, containing an extracellular amino-terminus and seven hydrophobic membrane spanning α-helices [3]. Among these receptors, there is a degree of sequence conservation within the putative transmembrane regions. The identification and characterization of new members of the receptor superfamily, taking advantage of their sequence similarities, would facilitate the understanding of functional domains and essential residues of the receptors and provide models for the G protein signaling systems.

*Corresponding author. Fax: (81) (6) 879-3579.

***Present address:* Research Institute for Molecular Genetics, Nara Medical University, Nara, Japan.

Abbreviations: G protein, guanine nucleotide-binding regulatory protein; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; cAMP, cyclic AMP; IP_3, inositol 1,4,5-triphosphate.

The nucleotide sequence data of GPCR01 and GPCR21 have been submitted to the DDBJ/EMBL/GenBank Data Libraries under accession numbers D21061 and D21062, respectively.

2. MATERIALS AND METHODS

2.1. *RNA preparation and polymerase chain reaction (PCR)*

Total RNA was prepared from mouse whole brain using the acid guanidinium thiocyanate–phenol–chloroform method [6] and poly(A)$^+$ RNA was selected using Oligotex-dT30 (Takara). First strand cDNA was synthesized with random hexamer and MMLV reverse transcriptase and then was subjected to PCR amplification using a set of degenerate oligonucleotide primers corresponding to conserved sequences of the third and sixth transmembrane domains among the known G protein-coupled receptors (TM3F: 5′-ATCCGAATTCTGTGIG(C/T)(G/C)ATC(A/G)(G/C)CITIGA-(C/T)(A/C)G(G/C)T; TM6R:5′-AGATGTCGACGAAGAAGGGC-(A/G)ICCA(G/A)CAG(A/C)II(A/G)(G/C/T)(A/G)AA, modified from the primers reported by Libert et al. [7]). The conditions of the PCR were as follows: 94°C, 1 min (denaturation); 55°C, 1 min (annealing); 72°C, 2 min (extension); 35 cycles. The amplified cDNA fragments were cloned into M13 phage vectors for sequencing. Out of 62 clones analyzed, two clones (MB10 and MBW5) were found to have unreported sequences with conserved receptor motifs (the sequence data of the full-length cDNA clone for the MB10 was submitted to the DDBJ/EMBL/GenBank Data Libraries under the accession number D17292).

2.2. *cDNA library screening and DNA sequencing*

In order to obtain the complete cDNA fragment for the clone MBW5, about 1×10^6 clones from a phage λgt10 mouse brain cDNA library were screened with the MBW5 cDNA fragment labeled with [α-^{32}P]dCTP by the random priming method [8]. Hybridization was performed at 53°C in 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1 × Denhardt's solution, 0.5% SDS and 150 μg/ml salmon sperm DNA. The filters were washed in 0.2 × SSC and 0.1%

SDS at 53°C and were autoradiographed. As a result, 11 positive clones were isolated. Restriction fragment length polymorphism analysis revealed the clone termed GPCR01 had a cDNA species different from the other 10 clones. The clone GPCR21 with the longest insert among the 10 clones and GPCR01 were subcloned into M13 phage vectors or Bluescript II plasmid vectors. Both strands of the cDNA clones were sequenced using Taq Dye Primer Cycle Sequencing Kit and 373A DNA autosequencer (Applied Biosystems).

2.3. *Northern blot analysis and reverse transcription-PCR (RT-PCR) analysis*

Total RNA and poly(A)$^+$ RNA were prepared from various mouse tissues in the method described in section 2.1 above. For Northern blot analysis, the RNA samples were electrophoresed on formaldehyde/0.9% agarose gels and transferred to Hybond-N nylon membranes (Amersham). The membranes were hybridized with ^{32}P-labeled GPCR01 or GPCR21 cDNA at 42°C in 50% formamide, 6 × SSC, 4 × Denhardt's solution, 1% SDS and 150 μg/ml salmon sperm DNA. The filters were washed at high stringency in a final wash of 0.2 × SSC, 0.1% SDS at 65°C. Two sets of oligonucleotide primers were synthesized for the analytical PCR amplification of reverse transcribed mRNAs for GPCR01 and GPCR21; GPCR01-F: 5'-GAACAACGCTGCCATCCTCT, and GPCR01-R: 5'-CTTGTGAGATCCAGTGCAAA; GPCR21-F: 5'-CACTCTCCAAGAACCATCTG (corresponding to 1064–1083; Fig. 1) and GPCR21-R: 5'-TCTGGAACATTCTAGAGCCA (corresponding to 1675–1694; Fig. 1). Total RNA samples were treated with RNase-free DNase I to avoid contamination of genomic DNA. First strand cDNA reverse transcribed from 100 ng of total RNA was amplified in a 5-μl reaction volume in the presence of [α-^{32}P]dCTP. The PCR run consists of 25 cycles of 45 s at 94°C, 60 s at 60°C and 60 s at 72°C. Each amplified sample was electrophoresed on 6% polyacrylamide gels. The gels were dried and exposed to Kodak X-Omat AR film.

2.4. *In situ hybridization analysis*

In situ hybridization was performed using ^{35}S-labeled cRNA probes as reported by Mizuno et al. [9]. 10 μm cryostat sections of adult mouse brains were hybridized with GPCR01 and GPCR21 riboprobes (3×10^6 cpm/ml) at 57°C in 50% formamide and 0.6 M NaCl, 1 × Denhardt's solution, 10% dextran sulfate and 100 μg/ml yeast tRNA and 100 μg/ml salmon sperm DNA. The sections were washed, treated with RNase A and then washed stringently in 2 × SSC at 55°C for 1 h, followed by 0.1 × SSC at 55°C for 3 h. The sections were exposed to X-ray film and then were dipped in Kodak NTB3 emulsion. Hybridization specificity was controlled using sense RNA probes.

2.5. *Eukaryotic expression of GPCR21*

The entire coding region of GPCR21 cDNA (corresponding to 493–1684; Fig. 1) was cloned into pRc/RSV vector (Invitrogen) and transfected using LipofectAMINE Reagent (BRL) into CHO-K1 cells. The transfectants were selected by G418 and were examined for the expression of GPCR21 by Northern blot analysis of cellular RNA. Cells were maintained in F-12 media with 10% fetal calf serum, kanamycin (50 μg/ml) and G418 (400 μg/ml) and incubated at 37°C in 5% CO_2 until use. CHO-K1 cells were provided by Japanese Cancer Resources Bank-Cell.

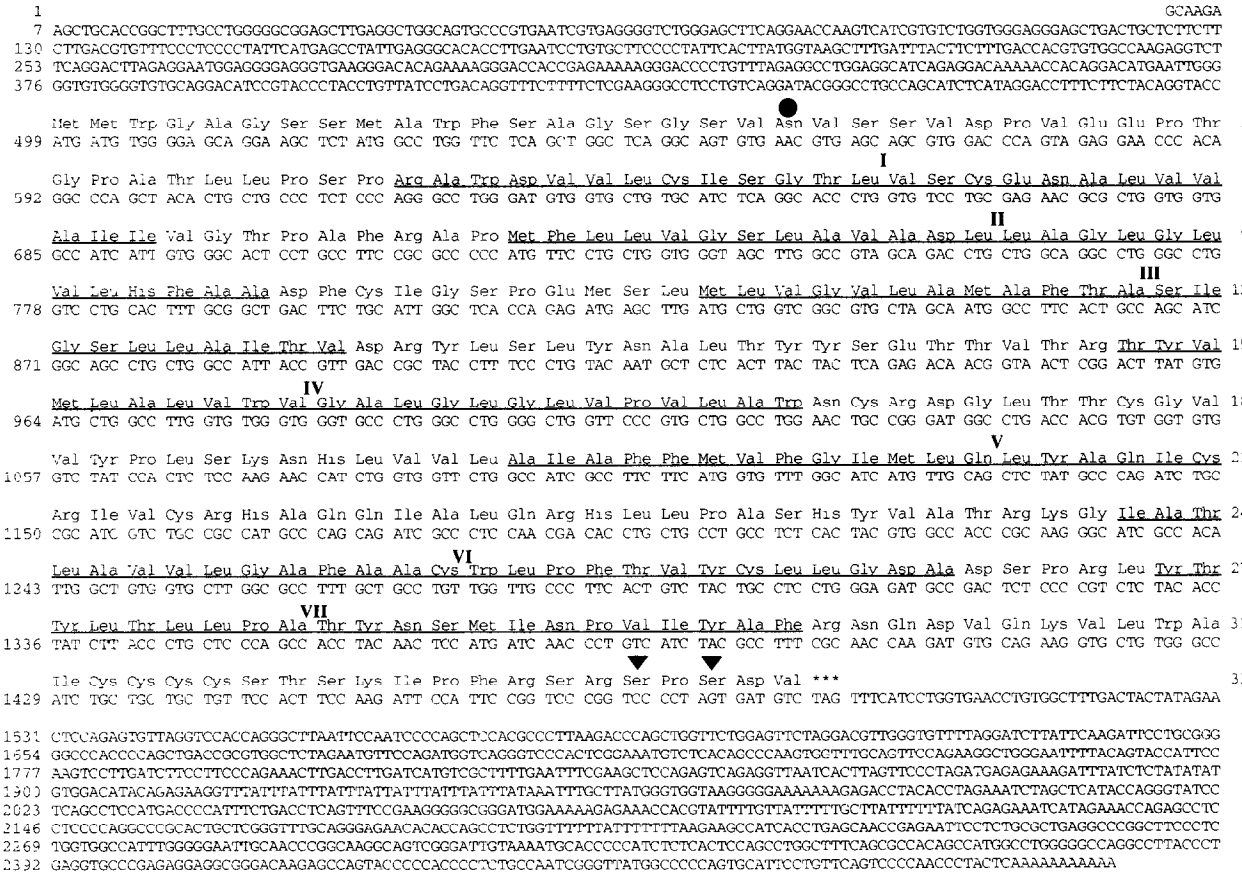

Fig. 1. Nucleotide and deduced amino amino acid sequence of putative GPCR21 receptor. Stop codon is indicated by ***. A potential N-linked glycosylation site in the NH_2-terminal region and phosphorylation sites in the COOH-terminal region are denoted by ● and ▼, respectively. The putative transmembrane domains I–VII are underlined and are assigned on the basis of a Kyte and Doolittle hydropathicity plot [11].

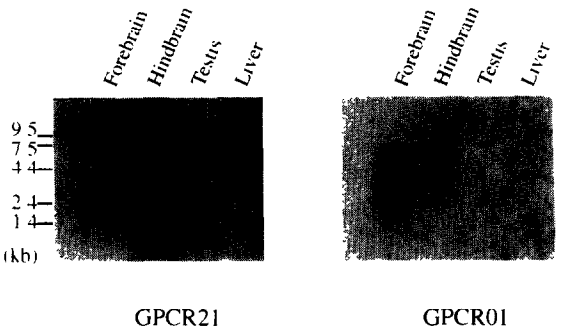

Fig. 2. RNA blot analysis of GPCR21 (left) and GPCR01 (right). Each lane contains 10 μg sample of poly(A)$^+$ RNA prepared from forebrain, hindbrain, testis and liver of adult male mouse. The film was exposed at −70°C for a week. The transcript of GPCR21 was detected as bands at 2.6 kb with approximately the same intensity both in forebrain and hindbrain. Messenger RNA for GPCR01 was observed predominantly in forebrain and to a lesser extent in hindbrain.

3. RESULTS AND DISCUSSION

In order to isolate new members of the G protein-coupled receptor family expressed in mouse central nervous system, RT-PCR was performed with poly(A)$^+$ RNA prepared from mouse whole brain. The amplified cDNA fragments were cloned into M13 vectors and were sequenced. The clone, termed MBW5, was found to have a 43-bp insert of unreported sequence with conserved receptor motifs. Screening of a mouse brain cDNA library with this partial cDNA fragment resulted in isolation of the full-length clone (GPCR21) for MBW5 as well as its homologous clone (GPCR01). Both clones were subjected to sequencing analysis on both strands.

The nucleotide sequence of clone GPCR21 specifies a 990-bp open reading frame coding for a 330-amino acid protein with a molecular weight of 35.5 kDa (Fig. 1). The presence of an in-frame stop codon in the 5′-upstream and the close match of the initiator ATG to the Kozak consensus sequence [10] indicates that the clone contains the entire coding region. According to hydropathicity analysis [11] (data not shown), the predicted polypeptide contains seven highly hydrophobic regions of 20–30 amino acids separated by stretches of more hydrophilic sequences, typical for the G protein-coupled receptor superfamily [3,12]. The receptor contains a potential *N*-linked glycosylation site at Asn^{20} [13] in the amino-terminal region. There are conserved proline residues in the fourth, sixth and seventh transmembrane domains (Pro^{171}, Pro^{262} and Pro^{294}). These residues producing a bend in the α-helical structure may be important in the binding pocket formation [14]. The presence of the consensus phosphorylation sites in the carboxyl-terminal region at Ser^{326} and Ser^{328} (calmodulin-dependent protein kinase sites) [15] suggests that the function of this receptor is modulated by phosphorylation.

Sequencing analysis of GPCR01 revealed that the longest open reading frame in the clone encodes a 334 amino acid residue protein. Homology searching of GPCR01 sequence on the GenBank database revealed that GPCR01 was the mouse counterpart of a previously reported rat orphan receptor R334 (91.4% and 89.7% identity in nucleotide sequence and amino acid sequence, respectively) [16]. GPCR21 and GPCR01 showed high sequence homology, 57.0% identity in amino acid sequence. The sequence identity between these receptors rises to 64.4% for the transmembrane residues, suggesting that the ligands for these receptors are likely to be identical or similar [17].

Northern blot analysis was performed to investigate the expression of GPCR21 in mouse tissues. As shown in Fig. 2, a mRNA species, approximately 2.6 kb in size, was detected in both forebrain and hindbrain. A faint band of the same size was also observed in testis. On the other hand, GPCR01 was expressed predominantly in forebrain and, to a lesser extent, in hindbrain. We also performed RT-PCR analysis using total RNA prepared from various mouse tissues (Fig. 3). The DNA fragments of 631 bp amplified from GPCR21 transcript were detected in various regions of brain and in testis but in no other tissues or organs examined. The intensive bands for GPCR01 (518 bp) were detected in frontal cortex, thalamus and hypothalamus. Although R334, the rat counterpart of GPCR01, was reported to be expressed in testis [16], our result showed that GPCR01 was expressed in liver rather than testis in mouse. The more precise distribution of mRNAs for these receptors in mouse brain was studied by in situ hybridization analysis (Fig. 4). GPCR21 was found to be expressed prominently in medial habenular nucleus and weaker signals were detected in cerebral cortex,

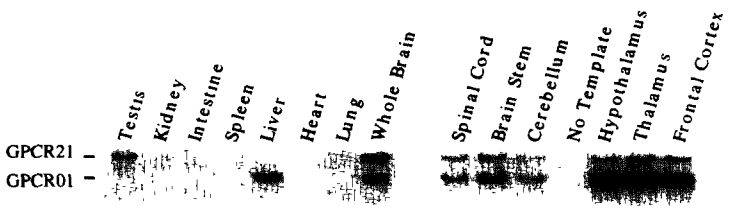

Fig. 3. RT-PCR analysis of GPCR21 and GPCR01. First strand cDNA reverse transcribed from total RNA prepared from various mouse tissues were amplified and then separated by electrophoresis on 6% polyacrylamide gels. The upper bands of 631 bp in size represent the expression of GPCR21, while the lower bands of 518 bp represent that of GPCR01.

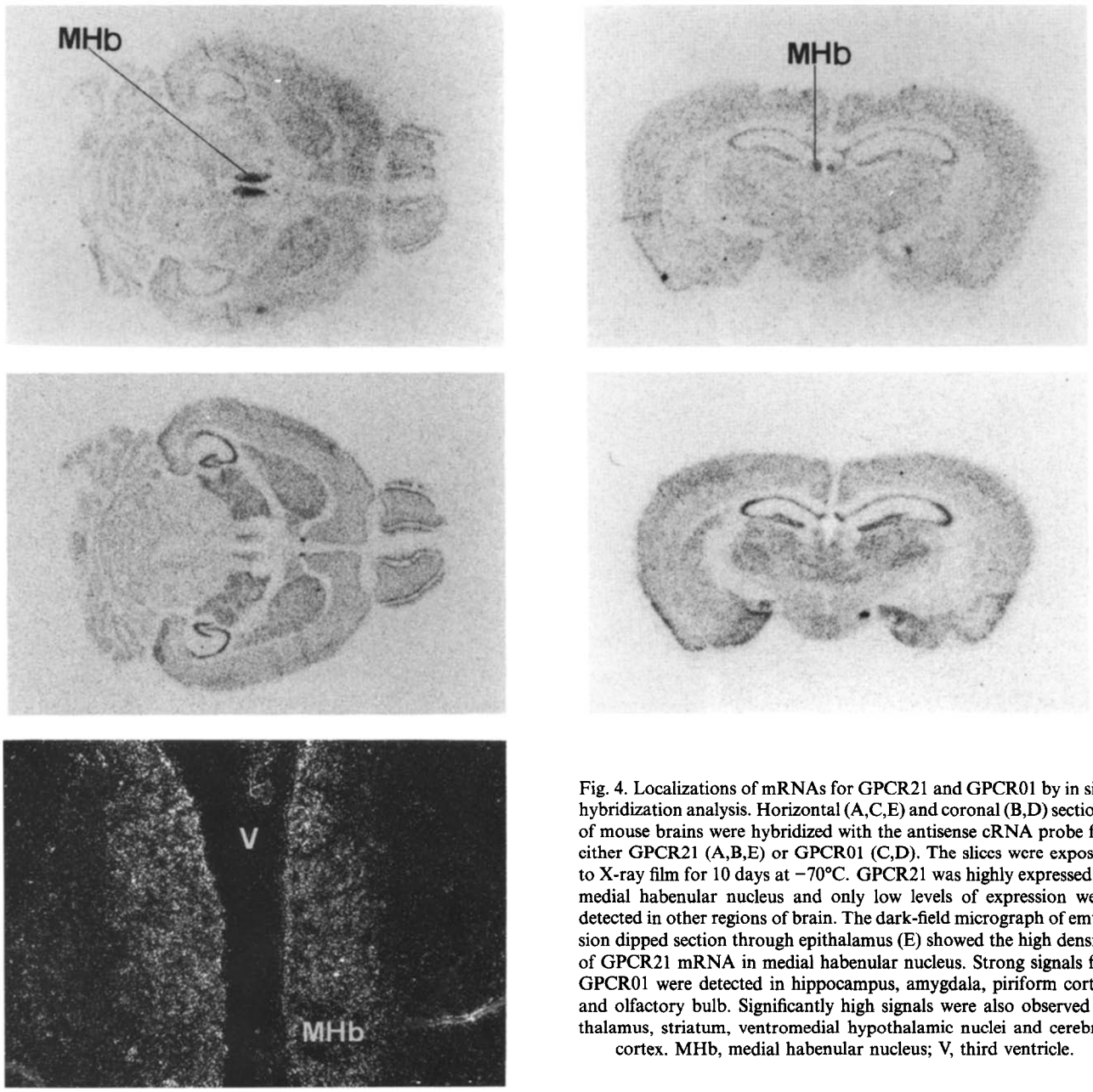

Fig. 4. Localizations of mRNAs for GPCR21 and GPCR01 by in situ hybridization analysis. Horizontal (A,C,E) and coronal (B,D) sections of mouse brains were hybridized with the antisense cRNA probe for either GPCR21 (A,B,E) or GPCR01 (C,D). The slices were exposed to X-ray film for 10 days at −70°C. GPCR21 was highly expressed in medial habenular nucleus and only low levels of expression were detected in other regions of brain. The dark-field micrograph of emulsion dipped section through epithalamus (E) showed the high density of GPCR21 mRNA in medial habenular nucleus. Strong signals for GPCR01 were detected in hippocampus, amygdala, piriform cortex and olfactory bulb. Significantly high signals were also observed in thalamus, striatum, ventromedial hypothalamic nuclei and cerebral cortex. MHb, medial habenular nucleus; V, third ventricle.

hippocampus, olfactory bulb and striatum. GPCR01, on the other hand, was expressed highly in hippocampus (granular cells of the dentate gyrus and CA2-3), amygdaloid nuclei, piriform cortex and olfactory bulb (external granular lamina and mitral cellular lamina). Significant levels of signals were also observed in thalamus, striatum, ventromedial hypothalamic nuclei and cerebral cortex.

Searching protein database revealed the highest homology of GPCR21 to the rat orphan receptor R334 (52.1% identity). In addition, GPCR21 showed significant similarities to melanocortin receptors (MSH receptor, 29.1%; ACTH receptor, 25.5% [4]), cannabinoid receptor (27.8%, [18]), adenosine receptors (A1, 26.0% [19]; A2a, 25.0% [7]; and A3, 28.8% [5]) and H2 histamine receptor (28.8% [20]). Multiple alignment of GPCR21 and GPCR01 with five other members of G protein-coupled receptors in shown in Fig. 5.

In order to identify a ligand for GPCR21, CHO-K1 cells stably expressing GPCR21 were tested for the changes of both cyclic AMP (cAMP) contents and inositol 1,4,5-triphosphate (IP_3) contents in response to various commercially available ligands. However, none of the substances examined affected the cAMP level of the IP_3 level.

We have obtained a novel cDNA clone encoding a

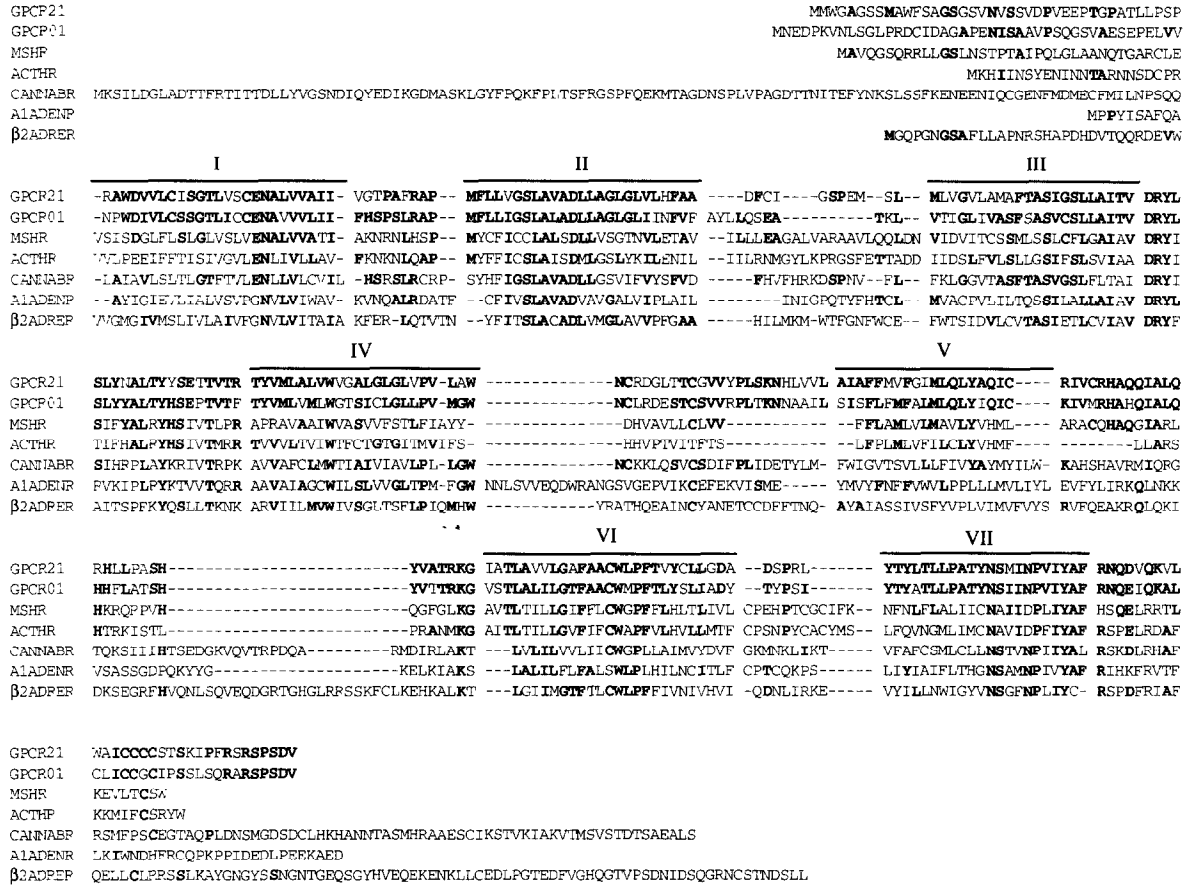

Fig. 5. Comparison of the amino acid sequences of the GPCR21, GPCR01 and five other members of G protein-coupled receptors (MSHR, human MSH receptor [4]; ACTHR, human ACTH receptor [4]; CANNABR, rat cannabinoid receptor [18]; A1ADENR, rat A1 adenosine receptor [19]; and β2ADRER, human β_2 adrenergic receptor [21]). Predicted transmembrane domains are numbered and indicated by a solid black line above the sequences. Gaps, indicated by hyphens, are introduced to obtain maximal homology. Amino acids identical to GPCR21 or GPCR01 are denoted in boldface.

putative mouse G protein-coupled receptor (GPCR21) along with its homologue (GPCR01). Based on the extremely high sequence similarity between them, we assume that their ligands are identical or closely related. Although the ligands for these receptors have not been identified, the predominant expression of their mRNAs in the limbic system of mouse brain suggest that they might be involved in a diverse set of behavioral functions, which include olfaction, ingestion, sexual behavior, endocrine function, autonomic function and brain stimulation. Since mRNA for GPCR21 was also detected in testis, it can be speculated that these receptors may play an important role in the neural regulation of sexual and/or reproductive functions. Thus, further characterization of these receptors, as well as the identification of their ligands, may contribute to our understanding of neuro-endocrine-behavioral modulation.

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REFERENCES

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–629.
[2] Birnbaumer, L., Abramowitz, J. and Brown, A.M. (1990) Biochim. Biophys. Acta 1031, 163–224.
[3] Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J. and Sealfon, S. (1992) DNA Cell Biol. 11, 1–20.
[4] Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D. (1992) Science 257, 1248–1251.
[5] Zhou, Q.Y., Li, C., Olah, M.E., Johnson, R.A., Stiles, G.L. and Civelli, O. (1992) Proc. Natl. Acad. Sci. USA 89, 7432–7436.
[6] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[7] Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.-J., Dumont, J.E. and Vassart, G. (1989) Science 244, 569–572.
[8] Feinberg, A.P. and Vogelstein, B. (1982) Anal. Biochem. 132, 6–13.
[9] Mzuno, R., Cavallaro, T. and Herbert, J. (1992) Invest. Ophthalmol. Vis. Sci. 33, 341–349.
[10] Kozak, M. (1986) Cell 44, 283–292.
[11] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[12] O'Dowd, B.F., Lefkowitz, R.J. and Caron, M.G. (1989) Annu. Rev. Neurosci. 12, 67–83.
[13] Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664.

[14] Applebury, M.L. and Hargrave, P.A. (1986) Vision Res. 26, 1881–1895.
[15] Kemp, B.E. and Pearson, R.B. (1990) Trends Biol. Sci. 15, 342–346.
[16] Eidne, K.A., Zabavnik, J., Peters, T., Yoshida, S., Anderson, L. and Taylor, P.L. (1991) FEBS Lett. 292, 243–248.
[17] Baldwin, J.M. (1993) EMBO J. 12, 1693–1703.
[18] Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C. and Bonner, T.I. (1990) Nature 346, 561–564.
[19] Mahan, L.C., McVittie, L.D., Smyk-Randall, E.M., Nakata, H., Monsma Jr., F.J., Gerfen, C.R. and Sibley, D.R. (1991) Mol. Pharmacol. 40, 1–7.
[20] Gantz, I., Munzert, G., Tashiro, T., Schaffer, M., Wang, L., DelValle, J. and Yamada, T. (1991) Biochem. Biophys. Res. Commun. 178, 1386–1392.
[21] Kobilka, B.K., Frielle, T., Dohlman, H.G., Bolanowski, M.A., Dixon, R.A., Keller, P., Caron, M.G. and Lefkowitz, R.J. (1987) J. Biol. Chem. 262, 7321–7327.