# 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

## Received 17 October 1993

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 proteincoupled 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  $\alpha$ -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 re-

\*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<sub>3</sub>, 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.

Published by Elsevier Science Publishers B.V.

ceptors and provide models for the G protein signaling systems.

# 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)<sup>+</sup> 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  $1 \times 10^6$  clones from a phage  $\lambda gt10$  mouse brain cDNA library were screened with the MBW5 cDNA fragment labeled with  $[\alpha$ -<sup>32</sup>P]dCTP by the random priming method [8]. Hybridization was performed at 53°C in  $6 \times SSC$  ( $1 \times SSC$  is 0.15 M NaCl plus 0.015 M sodium citrate),  $1 \times$  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)<sup>+</sup> 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 <sup>32</sup>P-labeled GPCR01 or GPCR21 cDNA at 42°C in 50% formamide, 6 × SSC,  $4 \times$  Denhardt's solution, 1% SDS and 150 µg/ml salmon sperm DNA. The filters were washed at high stringency in a final wash of  $0.2 \times SSC$ , 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'-GAA-CAACGCTGCCATCCTCT, and GPCR01-R: 5'-CTTGTGA-GATCCAGTGCAAA: GPCR21-F: 5'-CACTCTCCAAGAAC-CATCTG (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  $[\alpha$ -<sup>32</sup>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 <sup>35</sup>S-labeled cRNA probes as reported by Mizuno et al. [9]. 10  $\mu$ m cryostat sections of adult mouse brains were hybridized with GPCR01 and GPCR21 riboprobes (3 × 10<sup>6</sup> cpm/ml) at 57°C in 50% formamide and 0.6 M NaCl, 1 × Denhardt's solution, 10% dextran sulfate and 100  $\mu$ g/ml yeast tRNA and 100  $\mu$ g/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  $\mu$ g/ml) and G418 (400  $\mu$ g/ml) and incubated at 37°C in 5% CO<sub>2</sub> until use. CHO-K1 cells were provided by Japanese Cancer Resources Bank-Cell.

1 7 130 253	AGC! CTTX TCAC	IGCA GACG GGAC'	CCGG( TGTT TTAG)	CTTT TCCC AGGA	GCCT TCCC ATGG	GGGG( CTAT <sup>A</sup> AGGG(	GCGG. TCAT( GAGG(	AGCT GAGC( GTGA/	TGAG( CTAT AGGG	GCTG( TGAG( ACAC)	GCAG' GGCA( AGAA)	PGCCO CACCI AAGGO	CGTGI PTGAJ GACCI	AATCO ATCCI ACCGJ	STGA( IGTG( AGAA)	GGGG" CTTCC \AAGC	ICTGO CCCT# 3GACO	GGAGO ATTC <i>I</i> CCCTV	CTTC/ ACTT/ GTTT/	AGGAI ATGGI AGAGO	ACCAP FAAGC SCCTC	AGTCZ CTTT GGAGG	ATCGI SATTI SCATC	IGICI IACTI IAGAC	IGGT( ICTT) GACI	GGGAC IGACC	GGGAC CACGI	GCTGA IGTGC CAGGA	ACTGO GCCAJ ACATO	GCZ CTCT AGAGO GAAT	AAGA TCTT STCT NGGG	
376	GGT	GTGG	GGTG	TGCA	GGAC.	ATCC	GTAC	CCTA	CCTG	TATC	CTG	ACAG	STTT	CTTT.	ICTC	GAAGC	GCC.	ICCTO	<b>GTCA</b>	GAT	ACGGG	CCT	GCCAC	CATC	CTCA'	ragg/	ACCTI	<b>TCT</b>	ICTA(	CAGG	CACC	
	llet	Met	Trp	Gly	Ala	Gly	Ser	Ser	Met	Ala	Trp	Phe	Ser	Ala	Gly	Ser	Gly	Ser	Val	Asn	Val	Ser	Ser	Val	Asp	Pro	Val	Glu	Glu	Pro	Thr	31
499	ATG	ATG	TGG	GGA	GCA	GGA	AGC	TCT	ATG	GCC	TGG	TTC	TCA	GC'T	GGC	TCA	GGC	AGT	GTG	AAC	GTG	AGC	AGC	GTG	GAC	CCA	GTA	GAG	GAA	CCC	ACA	
																						1	ſ									
	Glv	Pro	216	Thr	Leu	Leu	Pro	Ser	Pro	Arg	Ala	Tro	Asp	Val	Val	Len	CVS	Tle	Ser	Glv	Thr	Leu	Val	Ser	(VS	Glu	Asn	Ala	Leu	Val	Val	62
592	aac	000	CCT	202	CTG	CTTC	CCC	TOT	000	200	CCC	TCC	CAT	GTG	OTO:	CTG	TOC	ATC:	TCA	COC	ACC	CTTC	OTG.	TTCC	TAC	GAG	AAC	ACG.	CTG	GTTG	GTG	
272	GGC	çça	901	ACA	C10	010	ccc	101	ccc	AGG	900	100	UNI	010	010	010	100	ні¢	ICH	000	1100	¢10	010	100	100	-	1410	000	010	010	0.0	
		<b>7</b> ].	<b>T</b> ] -	•••• 1	Q1		D		Dla a		<u>.</u> .	D		<b>D</b> 1	·	Ŧ	17.01	<u></u>	Com	1	210	1701	3.1.0	1.00	1	I, and	21-	c1	Loui	c1	T out	0.2
605	ALC	116	116	vdi	GLY	1111	P10	Ald	me	Arg	Ald	PIO	Net	File	orra	<u>neu</u>	orto	GLV	261	mmo	ALA	ODA	Ala	<u>aso</u>	- Leu	<u>DEG</u>	ALC .	GIV	000	019	Deu	30
685	GCC	ATC	A'1'1	GTG	GGC	AC'I'	CCT	GCC	TIC	CGC	GCC	CCC	AIG	TIC	CIG	CIG	GIG	GGT	AGC	TIG	GCC	GTA	GCA	GAC	CIG	CIG	GCA	GGC	CIG	GGC	CIG	
																	_		_		- •		_				-1		II	II.	- 1	
	Val	Leu	His	Phe	Ala	Ala	Asp	Phe	Cys	Ile	Gly	Ser	Pro	Glu	Met	Ser	Leu	Met	Leu	Val	GIV.	Va⊥	Leu	Ala	Met	Ala	Phe	Thr	Ala	Ser	Цe	124
778	GTC	CTG	CAC	TTT	GCG	GCT	GAC	TTC	TGĊ	ATT	GGC	TĊA	CCA	GAG	ATG	AGC	TTG	ATG	CTG	GTC	GGÇ	GTG	CTA	GCA	ATG	GCC	TTC	ACT	GCC	AGC	ATC	
	<u>Glv</u>	Şer	Leu	Leu	Ala	Ile	Thr	Val	Asp	Arg	Tyr	Leu	Ser	Leu	Tyr	Asn	Ala	Leu	Thr	Tyr	Tyr	Ser	Glu	Thr	Thr	Val	Thr	Arg	<u>Thr</u>	Tyr	Val	155
871	GGC	AGC	CTG	CTG	GCC	ATT	ACC	GTT	GAC	CGC	TAC	CTT	TCC	CTG	TAC	AAT	GCT	CTC	ACT	TAC	TAC	TCA	GAG	ACA	ACG	GTA	ACT	CGG	ACT	TAT	GTG	
							T	v																								
	Mer	Leu	Ala	Leu	Val	Tm	Val	Glv	Ala	Leu	Glv	Leu	Glv	Leu	Val	Pro	Val	Leu	Ala	Tro	Asn	Cvs	Arg	Asp	Glv	Leu	Thr	Thr	Cvs	Glv	Val	186
964	ZTTC	CTG	GCC	TTC:	CTC	TTCC	CTTC.	COT	000	CTC	000	CTTC	CCC	CTG	GTT	CCC	GTG	CTG	GCC	TGG	AAC	TGC	coc	GAT	222	CTG	ACC	ACG	TGT	GGT	GTG	
204	AIQ	010		110	010	100	010	001	000	010	000	010	000	010	011	000	010	010	000	100		100	000	<b></b>		,						
	Tra 1	· · · · ·	Deen	1	Com	T	3.00	II. e	Tau	Val	1/21	τ		T10	21-	Dho	Dho	Mot	17-1	Dho	C111	T10	Mot	tou	CIN	I ON	mur	31-	Cln	T10	Cure	217
	val	TAT	PIO	Leu	Ser	LYS	ASI	л15 010	Leu	vai	vai	Leu	ALC	110	ALC	PILE	Pile	Mec.	Vai	mmm	GIV	200	Amo	mmo	010	CIDC	1.4.1	ALG	GNO	110	mcc.	
1621	GIC	TAT	CCA	CIC	100	AAG	AAC	CAT	CIG	GIG	GTT	CIG	GUU	ATC	GCC	TIC	The	AIG	610	7.1.1.	GGC	ATC	AIG	1.1.G	CAG	CIC	.1.4.1	GUU	ÇAĢ	AIC	IGC	
		- 1		~				~ 1	~ 1	-	• •		~1			+				<b>a</b>	•• -	<b>.</b> .	**- 1	. 1 -	ml	<b>1</b>		a1	<b>T</b> 1 -		(D)=	240
	Arg	Ile	val	Cys	Arg	His	Ala	Gin	GIN	шe	Ala	Leu	GIN	Arg	His	Leu	Leu	Pro	Ala	Ser	His	ıγr	vai	Ala	Inr	Arg	Lys	GTY	TTG.	ALd	inr	248
1150	CGC	ATC	GTC	TGC	CGC	CAT	GCC	CAG	CAG	ATC	GCC	CTC	CAA	CGA	CAC	CTG	CTG	CCT	GCC	TCT	CAC	TAC	GTG	GCC	ACC	CGC	AAG	GGC	ATC	GCC	ACA	
	VI																															
	Leu	Ala	'/al	Val	Leu	<u>Gly</u>	Ala	Phe	Ala	Ala	Cvs	Trp	Leu	Pro	Phe	Thr	Val	Tyr	Cvs	Leu	Leu	Gly	Asp	Alā	Asp	Ser	Pro	Arg	Leu	<u>Tyr</u>	Thr	279
1243	TTG	GCT	GTG	GTG	CTT	GGC	GCĊ	$\mathbf{T}\mathbf{T}\mathbf{T}$	GCT	GCC	TGT	TGG	TTG	CCC	TIC	ACT	GTC	TAC	TGC	CTC	CTG	GGA	GAT	GCC	GAC	TCT	CCC	CGT	CTC	TAC	ACC	
							v	н																								
	TYY.	Leu	Thr	Leu	Leu	Pro	Ala	Thr	Tyr	Asn	Ser	Met	Ile	Asn	Pro	Val	Ile	Tyr	Ala	Phe	Arg	Asn	Gln	Asp	Val	Gìn	Lys	Val	Leu	Trp	Ala	310
1336	TAT	CTT	ACC	CTG	CTC	CCA	GCC	ACC	TAC	AAC	TCC	ATG	ATC	AAC	CCT	GTC	ATC	TAC	GCC	TTT	CGC	AAC	CAA	GAT	GTG	CAG	AAG	GTG	CTG	TGG	GCC	
																¥		T														
	Tle	Ovs	Cvs	Cvs	Cvs	Ser	Thr	Ser	Lvs	Ile	Pro	Phe	Ara	Ser	Ara	Ser	Pro	Ser	Asp	Val	* * *											330
1429	am	TICC	TGC .	man	TT-TT-	TCC	ACT	TCC	AAG	ATT	CCA	TT	CGG	TCC	CGG	TCC	CCT	AGT	GAT	GTC	TAG	TTT	CATTCO	TGGT	GAAC	CIGI	GGCI	TTGA	CTAC	TAT	GAA	
		100	100	100	τψτ	100		100						100																		
1521	CORCO.	12024	- Transmo	די ארייריי	TOC N		~~~~m	ידע היו	000.37	vince	0000	י הי היי	Vaca	COPT	12020	CC NO	erree	יייחיב	mcao	20000	PAGGA	COM	racar	CONTRA	mage	-2010-1	דיידאידי	Y AAC	איייאנ	CTG	200	
1000	0100	- AGA	37911	1400	10CA			0.001				. I UUI MIC NO				N N N N			100AC	22200	ncom			0111	2000		ית הי	TTAC	7700 A	CCAT	TOC	
1004	3300		LCCA(		1000c	2010		1 MUT 7 /	5191". Some	1 UUAU	1000 mo	10,000 10,000				CODC	0101			- 1990 1990 1990	0011	inna/	20110	CTO CTO	1,000	CACT	2202	, with the second	- NG TA			
1100	AAGI	ICCT.	IGAI	C I TU		_CAGA	ANAC:			SAICA	ALGIC .			44 PP			.CAGE	NUL DA	JOAGC	91 I MA	ALCAC	I TAC		L INC	10101	N INCO		1111 <i>1</i> 0	CONC	-1915		
1900	GTGC	JACA'	TACAC	JAGA	AGGT.	PIAT	ΓPAP.	I I'A'I'	FTAT.	rAT"I"	ATT	ATT	ATA	ATT	IGC IT	ATGC	GIG	TAAC	36666	SAAAr	AAAG	AGAC	CTAC	AUC'I	AGAA	ATCI	AGC1	CATA	ICCAG	0300	UCC .	
2023	TCAC	SCCIV	CATO	3ACC(	CAT	FICTO	JACC'	ICAG'	rrrco	GAAC	GGGC	GCGGC	GAIGC	JAAAA	AAGAC	SAAAC	CACO	FATT	PPIGI	TAT	TTTG	SC TTV	71-1-1-1	.1.1.A.I	CAGA	AGAA/	TCAT	AGAA	ACCA	GAGC	CIC	
2146	CICC	CCA	GCCC	CGCA	CTGC	rcgga	STTR	GCAGO	GAGA	FUCAC	CACCA	GCCI	CTGC	GTTT	PTTAI	TTTT	TTAF	AGAAG	GCCAT	ICACO	TGAG	CAAC	CGAC	AATI	CCTC	TGCC	SCTGA	GGCC	CGGC	TTCC	CTC	
2269	TGGI	rGGC	CATT	TGGG	GGAA'	rrgc/	AACCO	CGGCI	AAGGO	CAGTO	GGG7	TIG	TAAAA	ATGCA	ACCCC	CATC	TCTC	CACTO	CAGO	CTG	CTTI	CAGO	CGCCA	CAGC	CATC	GCCI	GGGG	GCCA	GGCC	TTAC	CCT	
2392	GAGO	STGC	CCGAC	GAGG	AGGC	GGGA	CAAGA	AGCCA	AGTAC	2000	ACCO	crer	GCC7	ATCO	GGT	ATGO	SCCCC	CAGI	IGCAI	rreei	IGTTĆ	AGTO	CCCP	ACCO	TACI	CAAA	AAAA	AAAA	<u>۱</u>			

Fig. 1. Nucleotide and deduced amino acid sequence of putative GPCR21 receptor. Stop codon is indicated by \*\*\*. A potential N-linked glycosylation site in the NH<sub>2</sub>-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  $10 \,\mu g$  sample of poly(A)<sup>+</sup> RNA prepared from forebrain, hindbrain, testis and liver of adult male mouse. The film was exposed at  $-70^{\circ}$ 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 proteincoupled 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 proteincoupled receptor superfamily [3,12]. The receptor contains a potential *N*-linked glycosylation site at Asn<sup>20</sup> [13] in the amino-terminal region. There are conserved proline residues in the fourth, sixth and seventh transmembrane domains (Pro<sup>171</sup>, Pro<sup>262</sup> and Pro<sup>294</sup>). These residues producing a bend in the  $\alpha$ -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<sup>326</sup> and Ser<sup>328</sup> (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  $-70^{\circ}$ 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 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.

Scarching 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 (Al, 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<sub>3</sub>) contents in response to various commercially available ligands. However, none of the substances examined affected the cAMP level of the IP<sub>3</sub> level.

We have obtained a novel cDNA clone encoding a

GPCP21 GPCP01 MSHP ACTHR CANNABR A1ADENP β2ADRER	MMGAGSSMAWFSAGSGSVNVSSVDPVEEPTGPATLLPSP MNEDPKVNLSGLPRDCIDAGAPENISAAVPSQGSVAESEPELVV MAVQGSQRRLLGSLNSTPTAIPQLGLAANQTGARCLE MKHIINSYENINNYTANNISDCPR MKSILLGLADTTFRTITTDLLYVGSNDIQYEDIKGDMASKLGYFPQKFPLTSFRGSPFQEKMTAGDNSPLVPAGDTINITEFYNKSLS5FKENEENIQCGENFMDMECFMLNPSQQ MPPVISAFQA MQPGNGSAFLLAPNRSHAPDHDVTQQRDEVw
	I II III
GPCR21 GPCP01 MSHR ACTHR CANNABP A1ADEMP β2ADREP	-RAWDVVLCISGTLVSCENALVVAII- VGTPAFRAP MFLLVGSLAVADLLAGLGUVLHPAADFCIGSPEMSL MLVGVLAMAPTASIGSLLAITV DRYL -NPWDIVLCSSGTLICCENAVVVLII- FHSPSLRAP MFLLIGSLALADLLAGLGUINFVF AYLLQSEATKL VFIGIVASFSASVCSLLAITV DRYL VSIBGEFLSLGUSLVENALVVATI- ARNENLHSP MYCFICCLALSDLLVSGTNVLETAVILLLEAGALVARAAVLQQLDN VIDVITCSSMLSSLCFLGAIAV DRYI -/LAIAVLSUFLGFF7/LENLIVLAV- FKNENLQAP MYFFICSLAISDLGSUFKUENILILLRMMGYLKPRGSFETTADD IDSTFVSLGSIFSLSVIAA DRYI -LAIAVLSUFLGFF7/LENLIVLCVIL -HSRSLRCRP- SYFFIGSLAVADLLGSVIFVYSFVFHVFHKDSPNFL FKLGGVTASFTASVGSLFLTAI DRYI -AYIGHSLIVLAVFGNVLVITAIA KFER-LQTVINYFITSLACADLWGLAVVPFGAAHILMKM-WFFGNFWCE FWTSIDVLCVTASIETLCVIAV DRYF
	IV V
GPCR21 GPCP01 MSHR ACTHR CANIABR A1ADEUR β2ADRER	SLYVALTYYSETTVTR TYVMLALVWVGALGLGUVPV-LAWNCRDGLTTCGVVYPLSKNHLVVL ALAFFMVFGIMLQLYAQIC RIVCRHAQQIALQ SLYYALTYHSEPTYFF TYVMLVMLWGTSICLGLLPV-MGWNCLRDESTCSVVRPLTKNNAALL SISFLFMFALMLQLYIQIC KIVMRHAHQIALQ SIFYALRYHSIVTLPR AFRAVAALWASVVFSTLFIAYYDHVAVLLCLVV
	VI VII
GPCR21 GPCR01 MSHR ACTHR CANNABR A1ADENR β2ADRER	RHLEPASH
GPCR21 GPCR01 MSHR ACTHP CAINABP A1ADENR ₿2ADPEP	WAICCCCSTSKIFFRSRSPSDV CLICCGCIPSSLSQRARSPSDV KEVLTCSX KKMIFCSRYW RSMFPSCBGTRQPLDISMGDSDCLHKHANNTASMHRAAESCIKSTVKIAKVIMSVSTDTSAEALS LKIMNDHFRCQPKPPIDEDLPEFKAED OELLCLPSSLKAYGNGYSSNGNTGROSGYHVEDOEKENKLLCEDLPGTEDFVGHQGTVPSDNIDSOGRNCSTNDSLL

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  $\beta_2$ ADRER, human  $\beta_2$  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.

Acknowledgements: We thank Dr. Kikuya Kato for the mouse brain cDNA library. We also thank Drs. Fumihisa Soga, Toyoki Maeda, Nobuyuki Takahashi, Yuji Nakatsuji and Yoichi Yamamoto for their advice and support.

## 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.