Discovery of a receptor related to the galanin receptors

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Abstract We report the isolation of a cDNA clone named GPR54, which encodes a novel G protein-coupled receptor (GPCR). A PCR search of rat brain cDNA retrieved a clone partially encoding a GPCR. In a library screening this clone was used to isolate a cDNA with an open reading frame (ORF) encoding a receptor of 396 amino acids long which shared significant identities in the transmembrane regions with rat galanin receptors GalR1 (45%), GalR3 (45%) and GalR2 (44%). Northern blot and in situ hybridization analyses revealed that GPR54 is expressed in brain regions (pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, frontal cortex, and striatum) as well as peripheral regions (liver and intestine). In COS cell expression of GPR54 no specific binding was observed for ^{125}I-galanin. A recent BLAST search with the rat GPR54 ORF nucleotide sequence recovered the human orthologue of GPR54 in a 3.5 Mb contig localized to chromosome 19p13.3.

Key words: G protein-coupled receptor; Polymerase chain reaction; Central nervous system expression; Chromosome

1. Introduction

Galanin is a 29–30 amino acid (aa) neuropeptide which is not a member of any known family of neuropeptides [1]. Galanin is expressed in both the central and peripheral nervous systems and modulates a wide variety of physiological processes including learning and memory [2], nociception [3], feeding [4], neurotransmitter and hormone release [5–7], and sexual behavior [8], and is considered to be involved in the pathogenesis of Alzheimer's disease [9]. The effects of galanin are mediated through at least three G protein-coupled receptors (GPCR), GalR1 [10], GalR2 [11], and GalR3 [12]. To date we have identified two human galanin receptors, GalR2 and GalR3 [13], both with distinct pharmacological profiles and tissue expression patterns. We now report a novel cDNA and gene GPR54, which encodes the closest known relative to the galanin receptor gene family.

2. Materials and methods

2.1. PCR amplification and cDNA library screening

A rat brain 5′ Stretch cDNA library (Clontech) and human genomic DNA were amplified by the polymerase chain reaction (PCR) using proofreading *Pfu* polymerase (Stratagene) and degenerate oligonucleotides based upon sequences encoding GPCR conserved transmembrane (TM) regions 3 (P1: 5′-CTGACCGGCATGABDETFGADCGHTA-3′; B = C or G, D = C or T, E = A or G or T, F = C or G or T, H = A or C, I = A or C or G or T, J = A or C or G, K = A or G) and 7 (P2: 5′-GAAGGCGTAGAFBAIJGGKTT). PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 55, 48, 45, 42, or 40°C for 40 s, and extension at 72°C for 30 s, for 30 cycles, followed by a 7 min extension at 72°C. The PCR products were extracted with phenol/chloroform, precipitated with ethanol and electrophoresed on a low melting point agarose gel. PCR product bands in the expected size range were excised from the gel, ligated into the *Eco*RV site of pBluescript SK$^-$ (Stratagene) and sequenced. One insert appeared to encode a novel GPCR and was labeled with [^{32}P]dCTP-α (NEN) by nick translation (Amersham) and used to screen the same library amplified above as previously described [14]. Positive phage clones were plaque purified and their inserts amplified by PCR using *Pfu* polymerase and primers flanking the λgt11 *Eco*RI cloning site. The PCR products were blunt-end ligated into the *Eco*RV site of pBluescript SK$^-$ (Stratagene) and sequenced on both strands.

2.2. Northern blot analysis

Rat mRNAs from several rat tissues were extracted as described previously [14]. Briefly, total RNA was extracted by the method of Chomczynski and Sacci [15] and poly(A)$^+$ RNA isolated using oligo(dT) cellulose spin columns (Pharmacia, Uppsala, Sweden). RNA was denatured and size fractionated on a 1% formaldehyde agarose gel, transferred onto nylon membrane and immobilized by UV irradiation. The blots were hybridized with a ^{32}P-labeled DNA fragment encoding GPR54, washed with 2×SSPE and 0.1% SDS at 50°C for 20 min and again with 0.1×SSPE and 0.1% SDS at 50°C for 2 h and exposed to X-ray film at −70°C in the presence of an intensifying screen.

2.3. In situ hybridization analysis

A ^{35}S-labeled DNA fragment encoding GPR54 was used as a probe for in situ hybridization. Preparation of rat brain sections and in situ hybridization procedures were done as previously described [16].

2.4. Expression of GPR54 cDNA in COS-7 mammalian cells

The African green monkey SV40 transformed kidney cell line (COS-7 cells), obtained from the American Type Culture Collection, was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Sigma), 50 U/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, McLean, VA), and 2 mM glutamine (Flow Laboratories) at 37°C under an atmosphere of 6% CO_2. 5×10^6 cells per 175 cm^2 culture flask were seeded in 20 ml of media and transiently transfected at 80% confluence with either 2.75, 5.5, or 11.65 μg of pcDNA3-GPR54 or pcIneo-hGALR1 plasmids and 70 μl of LipofectAMINE reagent (Life Technologies, Inc.), following recommendations of the manufacturer. Two days after transfection, cells were harvested following dissociation in enzyme-free dissociation solution (Specialty Media, Lavallette, NJ).

2.5. Membrane preparation and radioligand binding assays

Membranes were prepared from transfected cells by disruption by

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pressurized nitrogen cavitation in ice-cold membrane buffer (10 mM Tris, pH 7.4, 10 mM phenylmethylsulfonyl fluoride, 10 mM phosphoramidon). After a low speed (1100×*g* for 10 min at 4°C) and a high speed centrifugation (38 700×*g* for 15 min at 4°C), membranes were resuspended in buffer and their protein concentration determined (Bio-Rad assay kit). Binding of ^{125}I-human galanin (specific activity of 2200 Ci/mmol, DuPont NEN) was measured in membranes using a buffer of 25 mM Tris, pH 7.4, 0.3% BSA, 2 mM $MgCl_2$, 4 μg/ml phosphoramidon, and 10 μM leupeptin in a total volume of 250 μl. 200 pM of ^{125}I-human galanin was used. Reactions were initiated by the addition of membranes and the incubation was allowed to proceed at room temperature for 2 h. Non-specific binding was defined as the amount of radioactivity remaining bound in the presence of 10 μM unlabeled human galanin. Incubations were terminated by rapid filtration through GF/C filters which had been presoaked with 0.1% polyethylamine using a TOMTEC (Orange, CT) cell harvester.

3. Results and discussion

3.1. Cloning of GPR54

Based on sequence conservation of GPCR transmembrane regions, we employed a degenerate PCR strategy to search for novel genes and cDNAs encoding GPCRs. Two degenerate primers P1 and P2, based on conserved GPCR sequences in TM3 and TM7, respectively, were used to amplify human genomic DNA and an aliquot of a rat brain cDNA library with proofreading *Pfu* polymerase. Analysis of the PCR amplifications followed by gel electrophoresis demonstrated a strong ethidium bromide band of ~500 bp long. The DNA was excised, subcloned into the pBluescript vector, and sequenced to reveal a high level of GPCR amplification (Table 1). One of the resulting rat clones appeared to partially encode a galanin/opioid-like receptor. The partial cDNA was labeled with [^{32}P]dCTP-α and used to screen the cDNA library employed in the degenerate PCR. Two positive plaques were purified and their inserts amplified by PCR using *Pfu* polymerase and primers flanking the cloning site of the λgt11 vector. The PCR products were subcloned into pBluescript and sequenced. Sequence analysis revealed that each plaque encoded a region of a putative GPCR from TM3 to the carboxy-terminus identical to each other and the original probe. A second round of screening of 1×10^6 plaques freshly plated from the same library yielded an additional three positive plaques. PCR amplification of these positive plaques with λgt11 flanking primers, each paired with an internal primer, revealed that only one of these positive plaques contained the entire open reading frame (ORF). This plaque was purified, the insert subcloned into pBluescript and was confirmed to contain the 5′ end of the full-length open reading frame. Finally, two specific primers from the 5′ and 3′ ends of the ORF were used to amplify with *pfu* polymerase the full length rat cDNA 1.2 kb clone, named GPR54. Sequence analysis revealed the cloned GPR54 ORF to be identical to the previous phage clones and the original probe.

3.2. Sequence analysis of GPR54

GPR54 contained an ORF of 1191 bp encoding for a pro-

Table 1
Classification of products from degenerate primer-based PCR

Receptor	Rat whole brain cDNA			Human genomic DNA				Total
	45°C	42°C	40°C	55°C	48°C	45°C	40°C	
Adenosine A_2	0	1	0	0	0	0	0	1
Adrenergic α_1	2	3	1	0	0	1	0	7
Adrenergic β_3	0	0	0	0	1	0	0	1
Adrenomedullin	1	0	0	0	0	0	0	1
Cannabinoid 2	0	0	0	0	1	0	2	3
Chemokine CCR-6	0	0	0	1	1	0	0	2
Chemokine CCR-7	0	0	0	0	0	0	1	1
Chemokine CXCR-2	0	0	0	1	1	1	0	3
Chemokine CXCR-4	2	6	2	0	1	0	0	11
Dopamine D_5	0	0	0	0	0	0	1	1
Histamine H_2	0	1	0	0	0	0	0	1
Melanocortin 1	0	0	0	0	1	0	0	1
Olfactory	1	0	0	4	2	1	1	9
Purinoceptor P2U	0	0	0	0	0	0	1	1
Serotonin 5-HT_{5B}	1	0	0	0	0	0	0	1
Somatostatin SSTR1	7	33	0	0	0	0	0	40
Somatostatin SSTR2	6	3	1	0	3	0	0	13
Somatostatin SSTR3	0	1	1	0	0	0	1	3
APJ	1	0	0	1	0	0	0	2
OGR1	1	0	0	2	1	0	0	4
RDC1	16	1	2	1	1	2	0	23
GPR3	2	0	0	0	1	1	0	4
GPR4	4	0	0	0	0	0	0	4
GPR5	0	0	0	0	2	4	0	6
GPR6	0	0	0	0	1	0	0	1
GPR7	0	1	0	2	0	0	1	4
GPR8	0	0	0	0	0	1	0	1
GPR9	0	0	0	1	0	0	0	1
GPR25	0	0	0	1	0	1	0	2
GPR30	0	0	0	0	0	1	0	1
GPR54	1	0	0	0	0	0	0	1
No GPCR homology	63	4	11	4	16	5	10	113
Total	108	54	18	18	33	18	18	267

A tally of genes encoding various GPCRs amplified by PCR using degenerate oligonucleotide primers with different template and annealing temperature combinations. Our novel gene, GPR54, is shown in bold.

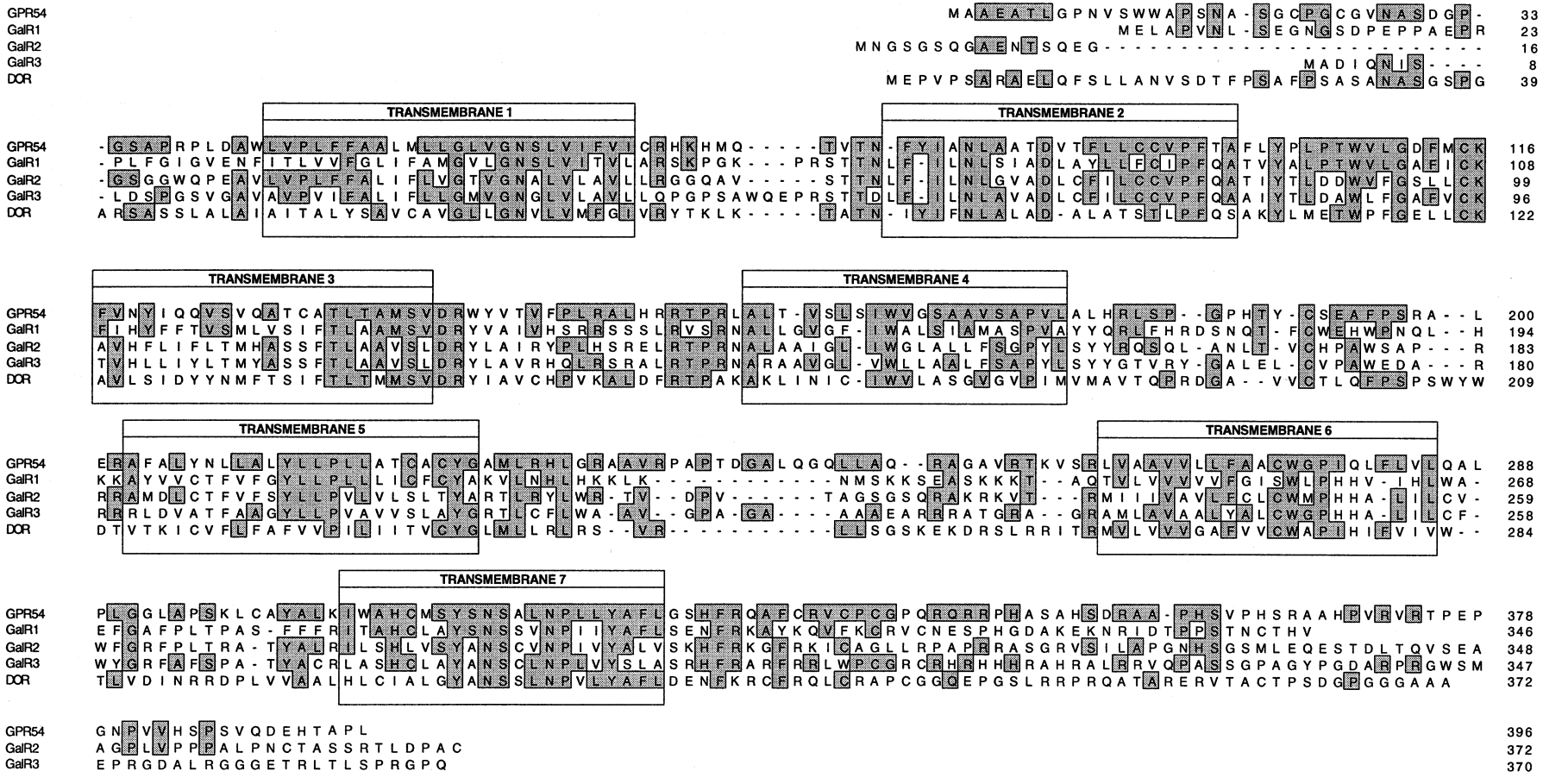

Fig. 1. An alignment of amino acid sequences of the rat GPR54 receptor with rat galanin receptors GalR1, GalR2, GalR3 and rat opioid receptor DOR. Conserved amino acids between GPR54 and the aligned receptors are shown boxed and shaded. Transmembrane regions are boxed and labeled. Numeric amino acid positions are indicated on the right. The GenBank accession number for rat GPR54 is AF115516.

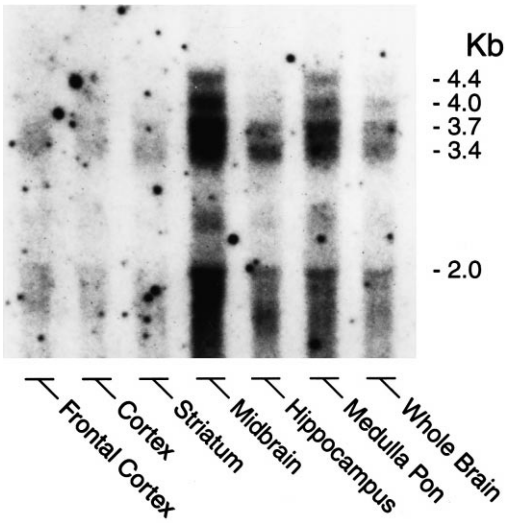

Fig. 2. Northern blot analysis of the tissue distribution of GPR54 mRNA in rat brain. Each lane contained 5 μg of poly(A)$^+$ RNA isolated from various rat tissues.

tein of 396 amino acids (aa). Using GPR54 in a BLAST search [17], the highest identity was observed with the galanin and opioid receptor families. Specifically, GPR54 shared an amino acid sequence identity in the TM regions with rat galanin receptors GalR1 (45%), GalR3 (45%), GalR2 (44%), and rat opioid receptor DOR (37%) (Fig. 1). Conserved residues and consensus sequences of the rhodopsin superfamily of GPCRs present in GPR54 included an asparagine in TM1, an aspartate in TM2, prolines in TMs 4–7, three consensus sequences for *N*-linked glycosylation in the amino-terminus, cysteines in the first and second extracellular loops, a PKA/PKC consensus sequence in the second intracellular loop, a PKC consensus sequence in the third intracellular loop and three possible palmitoylation cysteine sites in the carboxy tail. Significantly, various residues in the human GalR1 receptor shown to be important for high affinity galanin binding (corresponding to His-262, His-265, Glu-269, and Phe-280 in rat GalR1 [18,19]) were not conserved in GPR54. Among these, however, only His-262 is conserved among the three galanin receptors. In addition, the substitution of a tyrosine residue found in GPR54, GalR2 and GalR3 in place of Phe-280 in GalR1 was shown to have no significant effect on galanin binding [19] as opposed to previous studies where Phe-280 was replaced by alanine in GalR1 [18].

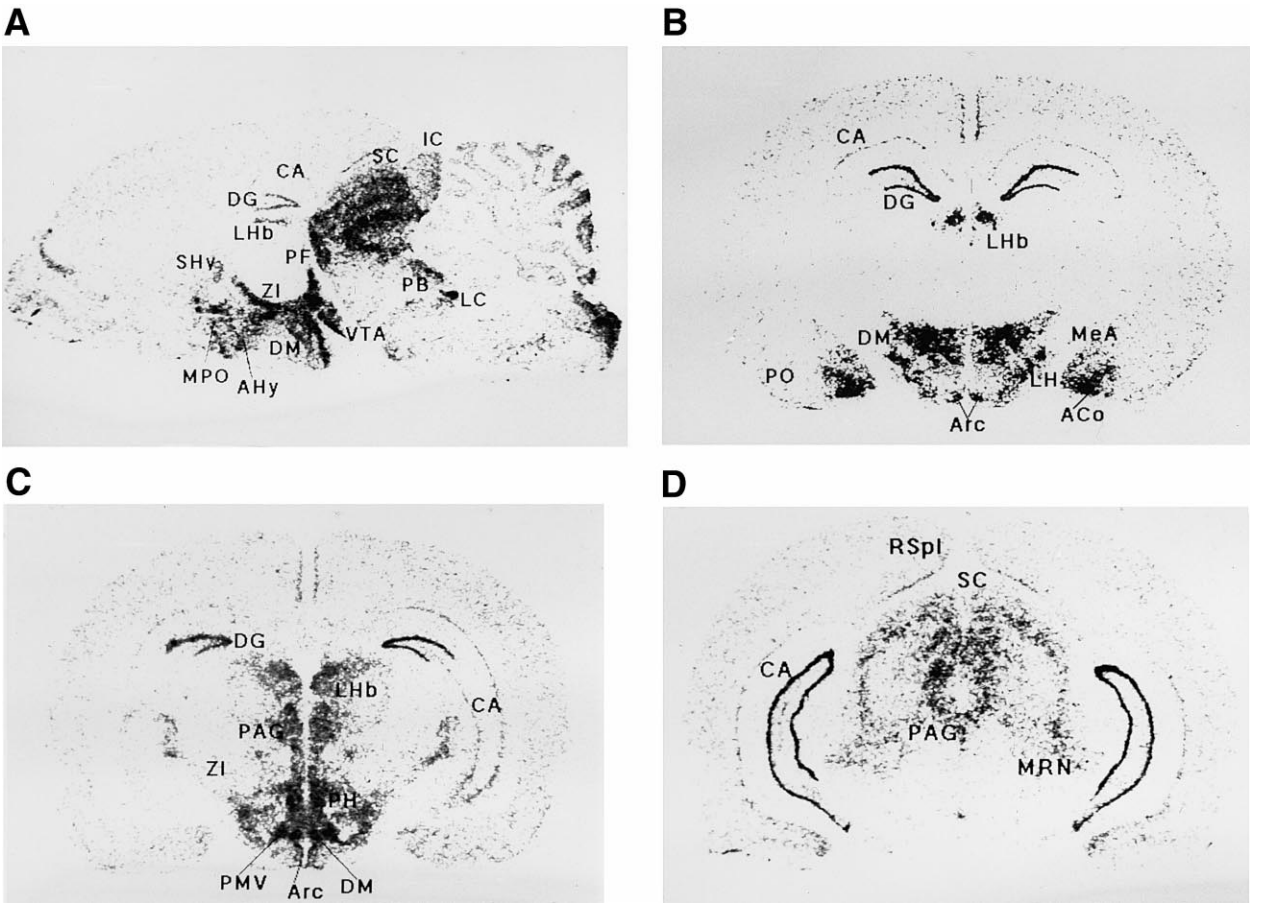

Fig. 3. Darkfield autoradiograms of sagittal and coronal sections of rat brain showing the localization of GPR54 receptor mRNA. A: A lateral representative section at 0.9 mm. Also shown are representitive sections at levels relative to the bregma at −3.3 mm (B), −3.8 mm (C) and −6.3 mm (D). Aco, cortical nucleus of the amygdala; AHy, anterior hypothalamic area; Arc, hypothalamic arcuate nucleus; IC, inferior colliculus; CA, field of Ammon's horn; DG, dentate gyrus; DM, dorsomedial hypothalamic nucleus; LC, locus coeruleus; LH, lateral hypothalamic area; LHb, lateral habenular nucleus; MeA, medial nucleus of the amygdala; MPO, medial preoptic area; MRN, mesencephalic reticular nucleus; PAG, periaqueductal gray; PB, parabrachial nucleus; PF, parafascicular thalamic nucleus; PH, posterior hypothalamic nucleus; PMV, ventral premamillary nucleus; PO, primary olfactory cortex; RSpl, retrosplenial cortex; SC, superior colliculus; SHy, septohypothalamic nucleus; VTA, ventral tegmental area; ZI, zona incerta.

3.3. Tissue distribution

Both Northern blot and in situ hybridization analyses of GPR54 were performed at high stringencies and with a DNA probe encoding GPR54 from TM3 to TM7 and with low identities to the genes encoding galanin and related receptors. The tissue distribution of GPR54 was obtained by northern blot analysis using poly(A)$^+$ RNA isolated from various rat tissues (Fig. 2). In the brain, multiple RNA transcripts with a complex pattern were detected in the medulla pons, midbrain, hippocampus, cortex, frontal cortex, and striatum. The most intense band was approximately 3.7 kb in length, with a single, larger transcript of approximately 12 kb length detected in the liver and intestine only. No transcripts were revealed in the cerebellum or kidney tissues. Future experiments will be required to determine the reasons underlying the multiple transcripts.

Using in situ hybridization of rat brain sections, the distribution of GPR54 mRNA was found to be discretely localized to many areas (Fig. 3). The highest levels of expression were seen in hypothalamic and amygdaloid nuclei. GPR54 mRNA was highly expressed in the zona incerta, ventral tegmental area, dentate gyrus, hypothalamic arcuate nucleus, dorsomedial hypothalamic nucleus, primary olfactory cortex, lateral habenular nucleus, lateral hypothalamic area, locus coeruleus, and the cortical and medial nuclei of the amygdala. GPR54 mRNA was also concentrated in the superior colliculus, medial preoptic area, anterior hypothalamic area, posterior hypothalamic nucleus, periaqueductal gray, parafascicular thalamic nucleus, parabrachial nucleus, and ventral premamillary nucleus. The signals detected in the septohypothalamic nucleus, inferior colliculus, medial nucleus of the amygdala, mesencephalic reticular nucleus and retrosplenial cortex were diffuse and less abundant.

GPR54's CNS expression pattern was found to resemble those of galanin receptors. Specifically, rat GalR1 mRNA expression is abundant in several brain regions including the hypothalamus, amygdala, hippocampus and locus coeruleus [20]. Rat GalR2 mRNA expression is found in the mamillary nuclei, the dentate gyrus and posterior hypothalamic and arcuate nuclei [13]. Rat GalR3 is found to be abundantly expressed in the CA regions of Ammon's horn and the dentate gyrus with transcripts also detected in thalamic, hypothalamic, mamillary and amygdaloid nuclei [13].

3.4. Pharmacological characterization of the receptor encoded by GPR54

The identity and overlapping expression patterns of GPR54 with the galanin receptors suggested that the encoded receptor may demonstrate binding to galanin. In preparation for the expression and binding studies, the 1.2 kb cDNA fragment encoding the ORF of GPR54 was subcloned into the multiple cloning site of the pcDNA3 expression vector and transiently transfected into COS-7 cells. No specific binding was observed with ^{125}I-human galanin. In contrast, specific and high affinity binding was observed under similar conditions with ^{125}I-human galanin in membranes prepared from COS cells transfected with human GalR1, as well as in our previous report for GalR2 and GalR3 [13].

3.5. Identification and chromosomal mapping of the human orthologue of GPR54

A BLAST search with the rat GPR54 sequence revealed high identity with a human 3.5 Mb contig located in chromosome 19p13.3 containing a serine protease gene cluster (Genbank accession number AC005379). Sequence analysis revealed a previously unrecognised 3.3 kb intron-containing human orthologue of GPR54 encoding a protein 398 aa in length and sharing a translated aa identity of 81% (100% identity in the TM regions). The genomic sequence revealed four introns located in TM2 ($\sim$800 bp, interrupting the translated FYI..ANL sequence), TM3 ($\sim$800 bp, interrupting IQQ..VSV), TM4 ($\sim$250 bp, interrupting WVG..SAA) and in the third intracellular loop ($\sim$180 bp, interrupting ALQ..GQV).

In summary, we report the cloning and chromosomal localization of a novel GPCR with complex and abundant CNS and peripheral expression. While preliminary studies have not shown specific binding between GPR54 and galanin, sequence identities and parallel distribution patterns between galanin receptors with GPR54 suggest that GPR54's endogenous ligand is peptidergic in nature.

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