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Research report

A human gene encodes a putative G protein-coupled receptor highly expressed in the central nervous system

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Abstract

The mammalian bombesin (Bn)-like neuropeptide receptors gastrin-releasing peptide receptor (GRP-R) and neuromedin B receptor (NMB-R) transduce a variety of physiological signals that regulate secretion, growth, muscle contraction, chemotaxis and neuromodulation. We have used reverse transcription-polymerase chain reaction (PCR) to isolate a cDNA from human brain mRNA, GPCR/CNS, that encodes a putative G protein-coupled receptor (GPCR) based upon the presence of the paradigmatic seven heptahelical transmembrane domains in its predicted amino acid sequence. Analysis of the deduced protein sequence of GPCR/CNS reveals this putative receptor to be 98% identical to the deduced amino acid sequence of a recently reported gene product and minimally identical (~23%) to both murine GRP-R and human endothelin-B (ET-B) receptor. Our deduced protein sequence differs at 12 positions, scattered throughout the open reading frame, relative to the original sequence. A 3.7 kb GPCR/CNS mRNA species is expressed in vivo in a tissue-specific manner, with highest levels detected in brain and spinal cord, lower levels found in testis, placenta and liver, but no detectable expression observed in any other tissue. Analysis of GPCR/CNS genomic clones reveals that the human gene contains one intron that is about 21 kb in length that divides the coding region into two exons and maps to human chromosome 7q31. No specific binding is observed with either a newly identified ligand (DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14)) having high affinity for all Bn receptor subtypes or Bn after GPCR/CNS is stably expressed in fibroblasts. No elevation in inositol trisphosphate is observed after the application of micromolar levels of either DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) or Bn, a concentration of agonist known to activate all four known Bn receptor subtypes. When GPCR/CNS is expressed in *Xenopus* oocytes, no activation of the calcium-dependent chloride channel is detected despite the addition of micromolar levels of Bn peptide agonists. We conclude that the natural ligand for this receptor is none of the known naturally occurring Bn-like peptides and the true agonist for GPCR/CNS remains to be elucidated. © 1998 Elsevier Science B.V.

Keywords: G protein-coupled receptor; Orphan receptor; Substantia nigra; Corpus callosum

1. Introduction

G protein-coupled receptors (GPCRs) are a large superfamily of receptors that transduce extracellular signals into cytoplasmic responses by activating heterotrimeric GTP-binding proteins (G proteins) which in turn modulate the activities of adenyl cyclase, phospholipase C, or ion channels. Very often, a subfamily of several distinct GPCRs exist that bind a particular class of agonists. Over the last 6 yr, four structurally and pharmacologically distinct GPCRs which mediate signalling by bombesin (Bn)-like peptides have been cloned and characterized: GRP-R or bb2 [1,13], NMB-R or bb1 [15], Bn receptor subtype 3 (BRS-3) or bb3 [7,6] and Bn receptor subtype 4 (BB4) [9]. Three of the four receptors (GRP-R, NMB-R and BRS-3) are known to exist in mammals, while, at present, BB4 has only been identified in frogs. All four receptors activate a pertussis toxin insensitive heterotrimeric G protein which in turn

Abbreviations: bp, base pair; Bn, Bombesin; BRS-3, bombesin receptor subtype-3; dNTPs, deoxynucleotide triphosphates; ET-B, endothelin-B; GPCR, G protein-coupled receptor; GPCR/CNS, G protein-coupled receptor/central nervous system; GRP-R, gastrin-releasing peptide receptor; kb, kilobase; ml, milliliter; NMB-R, neuromedin B receptor; PCR, polymerase chain reaction

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activates phospholipase C. Three of the four receptors (GRP-R, NMB-R, BB4) bind one or more of the known, naturally occurring Bn-like peptides (GRP, NMB, or Bn) with high affinity (K_d in the nanomolar range). BRS-3 has low affinity ($K_i > 1$ micromolar) for the naturally occurring Bn-like peptides GRP, NMB and Bn [8].

Predicted amino acid sequence comparison of the four receptors show these receptors to be between 48% and 62% identical, typical for neuropeptide receptor subtypes that bind an overlapping spectrum of ligands. In addition, the genomic structures of the three mammalian Bn receptors are very similar, with two introns dividing the receptor coding region into three exons at identical locations within the receptor sequence. In this report, we further characterize a human gene encoding a member of the GPCR superfamily, whose amino acid sequence shows high sequence identity (~98%) to an orphan GPCR recently described [16] and limited sequence identity (~23%) with the murine GRP-R and human endothelin-B receptor [11], when compared to sequences found in GenBank.

2. Materials and methods

2.1. cDNA synthesis and PCR amplification

Poly A^+ RNA (0.5 μg) isolated from human fetal brain (Clontech) was used as template for the synthesis of cDNA using random hexamer primers (Boehringer Mannheim). After incubating the Poly A^+ RNA and random primers (100 ng) at 70°C for 10 min, 0.4 U/μl RNAsin (Promega), 10 mM dithiothreitol, 0.4 mM deoxynucleotide triphosphates (dNTPs), 10 U/μl Superscript II reverse transcriptase (Life Technologies) and 1X reverse transcriptase buffer were added and incubated at 25°C for 10 min. The cDNA synthesis reaction was then incubated at 50°C for an additional 45 min followed by 10 min at 950°C to heat inactivate the enzyme.

PCR reactions were performed in 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP and included 1.25 units of Pfu DNA polymerase (Stratagene), 0.20 μg of a sense primer, 0.20 μg of an antisense primer and 2% of the cDNA reaction as template. The cDNA was amplified using gene-specific oligonucleotide primers that amplified the open reading frame of a cDNA whose sequence was submitted in Patent Application PCT/US94/11843. The sense primer is 5′ GTGCCCTCACCAAGCCATGCG 3′ and the antisense primer is 5′ GTCCTTCAGCAATGAGTTCCGAC 3′. Samples were subjected to 40 cycles of PCR amplification using a Perkin–Elmer 9600 thermocycler. The PCR reactions were initially denatured at 95°C for 40 s, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 66°C for 30 s and extension at 72°C for 2 min. To facilitate ligation into the pCR2.1 vector (Invitrogen), deoxyadenosine was added to the 5′ ends of the PCR reaction products by adding 1 μl of Taq polymerase (Perkin–Elmer) per 50 μl PCR reaction and incubating at 75°C for 10 min. The predicted 1.8 kb PCR product was excised from a 1% low-melt agarose gel (FMC) and then purified using the Wizard PCR Preps DNA Purification System (Promega) and ligated into the pCR2.1 vector. For expression studies, the insert was released from the pCR2.1 vector using *Hin*dIII and *Xba*I restriction endonucleases and directionally cloned into the multiple cloning site of *Hin*dIII- and *Xba*I-digested pcDNA3 vector (Invitrogen).

2.2. cDNA and P1 sequence analysis

Plasmid DNA was purified using a Wizard Miniprep Kit (Promega) and both strands of the cDNA fragment were sequenced by the dideoxynucleotide chain termination method using an automated Applied Biosystems Model 373A DNA sequencer or manually using a *fmol* DNA Sequencing System (Promega). The nucleic acid and deduced protein sequences were compared to sequences in the database server at the National Center for Biotechnology using the Blast network service. Protein sequences were aligned using the University of Wisconsin GCG package and displayed using Microsoft Excel 5.0a. P1 human genomic clones (GS Control Numbers 13880, 14014; Genome Systems,) were sequenced using Promega's *fmol* DNA Sequencing System. Extra long PCR amplification of P1 DNA was performed using the GeneAmp XL PCR kit (Perkin–Elmer) according to manufacturer's instructions. An aliquot of the amplification mixture was subjected to electrophoresis in a 0.5% SeaKem Gold (FMC) agarose gel and the DNA visualized by ethidium bromide staining.

2.3. Generation of G418-selected stable clones

A clonal mouse fibroblast cell line (BALB/B1) was obtained by re-cloning the BALB 3T3 cell line (American Type Culture Collection) and grown at 37°C in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (v/v) qualified fetal bovine serum (Life Technologies) and a 1:200 dilution of a penicillin–streptomycin solution (Life Technologies). The cells were expanded by detachment using trypsin-EDTA (Life Technologies) treatment and subculturing at a split ratio of 1:20 every 3 to 4 days. BALB/B1 cells were transfected with 3 μg of plasmid DNA by 18 μg Lipofectamine (Life Technologies) in Opti-MEM (Life Technologies) for 1 h at 37°C. Twenty-four hours post transfection, the cells were released from 10 cm^2 culture dishes by treatment with trypsin-EDTA and seeded at varying densities ranging from 100–10,000 cells/10 cm^2 dish. Forty-eight hours post transfection the cells were placed in medium containing 900 μg/ml G418 and selection continued for 10 to 14 days. Clones were screened for GPCR/CNS mRNA expression by Northern blot analysis.

2.4. RNA isolation and Northern blot analysis

Cells were harvested by scraping the monolayer in 1 ml of PBS and pelleting the cells at 1500 rpm for 5 min. Total RNA was isolated using RNeasy Total RNA kit (Qiagen) according to the manufacturer's instructions. RNA concentrations were calculated by measuring UV absorbance at 260 nM. Each RNA sample (10 μg) was denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of intact 28S and 18S rRNA. RNA was blotted onto Zetabind nylon membranes (Cuno) and cross-linked by UV irradiation using a Stratalinker (Stratagene). A 1.8-kb *Hin*dIII/*Xba*I GPCR/CNS cDNA fragment containing the entire 613 amino acid open reading frame was radiolabelled using the Random Primers DNA Labelling System (Life Technologies). Verification that similar amounts of RNA were applied to each lane was performed by rehybridizing the membranes with a human β-actin cDNA insert. The β-actin cDNA insert was a 931-bp PCR product generated using oligonucleotide primers described elsewhere [3]. Hybridization and membrane washing conditions were as described previously [4]. The transfected cell line showing highest levels of GPCR/CNS expression (GPCR/CNS-23) was used for binding and inositol phosphate assays.

2.5. Whole cell radioligand binding assays

Competitive binding assays using the tracers ^{125}I-[Tyr^4]Bn (2200 Ci/mmol) or ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) (2200 Ci/mmol) [10] were performed as described previously [2]. Briefly, disaggregated transfected BALB/B1 fibroblasts (8×10^6 cells/ml) were incubated for 45 min at room temperature in a modified HEPES buffer containing 0.16 mg/ml soybean trypsin inhibitor (Sigma), 0.14% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin (Sigma), 10 μM [4-(2-aminoethyl)-benzenesulfonylflouride hydrochloride] (AEBSF; ICN), 250,000 cpm/ml tracer with or without 1 μM unlabelled Bn or [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) peptide. Bound ligand is separated from unbound by pelleting the cells through an oil phase. Radioactivity measurements were determined using a Cobra II gamma counter (Packard).

2.6. Measurement of inositol phosphates

BALB/B1 transfected cells were stimulated with either 10 μM Bn or 10 μM [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) and total phosphoinositides determined as described [2]. Briefly, cells were seeded at 30,000 cells/well in a 24 well dish and the following day the serum in the culture medium was reduced from 10% to 0.25%, to induce a relatively quiescent cell population. Forty-eight hours after seeding the cells, fresh medium containing 0.25% serum and 3 μCi/ml *myo*-[2-^{3}H(N)inositol (Dupont) was added for 24 h. The cells were washed and incubated in a modified HEPES buffer containing 20 mM LiCl for 30 min and then incubated for 20 min at 37°C with either Bn or [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14). The reaction was stopped by addition of 0.5% HCl in methanol and total ^{3}[H]inositol phosphates isolated by anion exchange chromatography.

2.7. Xenopus laevis oocyte expression studies

Oocytes were prepared, maintained, injected with in vitro transcripts and monitored for receptor-mediated signal transduction as described [12]. Briefly, defolliculated oocytes are microinjected with 50 nl of in vitro transcript (20 μg/ml) encoding either GPCR/CNS or mGRP-R. One day later, the two-electrode voltage clamp technique is used for electrophysiological analysis of receptors expressed by injected oocytes. The oocytes are voltage clamped at -60 mV and agonists applied. Receptor activation of phospholipase C results in inositol trisphosphate generation, which in turn releases calcium from intracellular stores. Elevation in intracellular calcium triggers the opening of a calcium-dependent chloride channel endogenous to the oocyte membrane. The restoring current needed to balance the outward flow of chloride through this channel is recorded as a tracing of current flowing through the electrode vs. time.

2.8. Chromosomal localization

Briefly, P1/13880 DNA was labelled with digoxigenin dUTP by nick translation and hybridized to normal metaphase lymphocyte chromosomes as performed by Genome Systems [14]. Specific fluorescent in situ hybridization signals were detected by incubating the slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAP1.

3. Results

3.1. Analysis of GPCR/CNS cDNA and P1 genomic clones

The nucleotide sequence of a GPCR/CNS cDNA clone is shown in Fig. 1. The cDNA contained a long open reading frame beginning at nucleotide 23 that encodes a protein of 613 amino acids with a predicted molecular mass of 67,113 Da and p*I* of 8.68. The presumed initiating AUG is the first methionine codon. Hydropathy analysis of the predicted GPCR/CNS protein reveals the characteristic seven stretches of hydrophobic amino acids, consistent with a seven transmembrane structure typical of GPCRs (data not shown). Two overlapping human P1 clones were

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                                                            TGTGCCCTCACCAAGCC      17

     ATGCGAGCCCCGGGCGCGCTTCTCGCCCGCATGTCGCGGCTACTGCTTCTGCTACTGCTC     77
1    M  R  A  P  G  A  L  L  A  R  M  S  R  L  L  L  L  L  L  L

     AAGGTGTCTGCCTCTTCTGCCCTCGGGGTCGCCCCTGCGTCCAGAAACGAAACTTGTCTG    137
21   K  V  S  A  S  S  A  L  G  V  A  P  A  S  R  N  E  T  C  L

     GGGGAGAGCTGTGCACCTACAGTGATCCAGCGCCGCGGCAGGGACGCCTGGGGACCGGGA    197
41   G  E  S  C  A  P  T  V  I  Q  R  R  G  R  D  A  W  G  P  G

     AATTCTGCAAGAGACGTTCTGCGAGCCCGAGCACCCAGGGAGGAGCAGGGGGCAGCGTTT    257
61   N  S  A  R  D  V  L  R  A  R  A  P  R  E  E  Q  G  A  A  F

     CTTGCGGGACCCTCCTGGGACCTGCCGGCGGCCCCGGGCCGTGACCCGGCTGCAGGCAGA    317
81   L  A  G  P  S  W  D  L  P  A  A  P  G  R  D  P  A  A  G  R

     GGGGCGGAGGCGTCGGCAGCCGGACCCCCGGGACCTCCAACCAGGCCACCTGGCCCCTGG    377
101  G  A  E  A  S  A  A  G  P  P  G  P  P  T  R  P  P  G  P  W

     AGGTGGAAAGGTGCTCGGGGTCAGGAGCCTTCTGAAACTTTGGGGAGAGGGAACCCCACG    437
121  R  W  K  G  A  R  G  Q  E  P  S  E  T  L  G  R  G  N  P  T

     GCCCTCCAGCTCTTCCTTCAGATCTCAGAGGAGGAAGAGAAGGGTCCCAGAGGCGCTGGC    497
141  A  L  Q  L  F  L  Q  I  S  E  E  E  E  K  G  P  R  G  A  G

     ATTTCCGGGCGTAGCCAGGAGCAGAGTGTGAAGACAGTCCCCGGAGCCAGCGATCTTTTT    557
161  I  S  G  R  S  Q  E  Q  S  V  K  T  V  P  G  A  S  D  L  F

     TACTGGCCAAGGAGAGCCGGGAAACTCCAGGGTTCCCACCACAAGCCCCTGTCCAAGACG    617
181  Y  W  P  R  R  A  G  K  L  Q  G  S  H  H  K  P  L  S  K  T

     GCCAATGGACTGGCGGGGCACGAAGGGTGGACAATTGCACTCCCGGGCCGGGCGCTGGCC    677
201  A  N  G  L  A  G  H  E  G  W  T  I  A  L  P  G  R  A  L  A

     CAGAATGGATCCTTGGGTGAAGGAATCCATGAGCCTGGGGGTCCCCGCCGGGGAAACAGC    737
221  Q  N  G  S  L  G  E  G  I  H  E  P  G  G  P  R  R  G  N  S

     ACGAACCGGCGTGTGAGACTGAAGAACCCCTTCTACCCGCTGACCCAGGAGTCCTATGGA    797
241  T  N  R  R  V  R  L  K  N  P  F  Y  P  L  T  Q  E  S  Y  G

     GCCTACGCGGTCATGTGTCTGTCCGTGGTGATCTTCGGGACCGGCATCATTGGCAACCTG    857
261  A  Y  A  V  M  C  L  S  V  V  I  F  G  T  G  I  I  G  N  L

     GCGGTGATGAGCATCGTGTGCCACAACTACTACATGCGGAGCATCTCCAACTCCCTCTTG    917
281  A  V  M  S  I  V  C  H  N  Y  Y  M  R  S  I  S  N  S  L  L

     GCCAACCTGGCCTTCTGGGACTTTCTCATCATCTTCTTCTGCCTTCCGCTGGTCATCTTC    977
301  A  N  L  A  F  W  D  F  L  I  I  F  F  C  L  P  L  V  I  F

     CACGAGCTGACCAAGAAGTGGCTGCTGGAGGACTTCTCCTGCAAGATCGTGCCCTATATA   1037
321  H  E  L  T  K  K  W  L  L  E  D  F  S  C  K  I  V  P  Y  I

     GAGGTCGCTTCTCTGGGAGTCACCACTTTCACCTTATGTGCTCTGTGCATAGACCGCTTC   1097
341  E  V  A  S  L  G  V  T  T  F  T  L  C  A  L  C  I  D  R  F

     CGTGCTGCCACCAACGTACAGATGTACTACGAAATGATCGAAAACTGTTCCTCAACAACT   1157
361  R  A  A  T  N  V  Q  M  Y  Y  E  M  I  E  N  C  S  S  T  T

     GCCAAACTTGCTGTTATATGGGTGGGAGCTCTATTGTTAGCACTTCCAGAAGTTGTTCTC   1217
381  A  K  L  A  V  I  W  V  G  A  L  L  L  A  L  P  E  V  V  L

     CGCCAGCTGAGCAAGGAGGATTTGGGGTTTAGTGGCCGAGCTCCGGCAGAAAGGTGCATT   1277
401  R  Q  L  S  K  E  D  L  G  F  S  G  R  A  P  A  E  R  C  I

     ATTAAGATCTCTCCTGATTTACCAGACACCATCTATGTTCTAGCCCTCACCTACGACAGT   1337
421  I  K  I  S  P  D  L  P  D  T  I  Y  V  L  A  L  T  Y  D  S

     GCGAGACTGTGGTGGTATTTTGGCTGTTACTTTTGTTTGCCCACGCTTTTCACCATCACC   1397
441  A  R  L  W  W  Y  F  G  C  Y  F  C  L  P  T  L  F  T  I  T

     TGCTCTCTAGTGACTGCGAGGAAAATCCGCAAAGCAGAGAAAGCCTGTACCCGAGGGAAT   1457
461  C  S  L  V  T  A  R  K  I  R  K  A  E  K  A  C  T  R  G  N

     AAACGGCAGATTCAACTAGAGAGTCAGATGAACTGTACAGTAGTGGCACTGACCATTTTA   1517
481  K  R  Q  I  Q  L  E  S  Q  M  N  C  T  V  V  A  L  T  I  L

     TATGGATTTTGCATTATTCCTGAAAATATCTGCAACATTGTTACTGCCTACATGGCTACA   1577
501  Y  G  F  C  I  I  P  E  N  I  C  N  I  V  T  A  Y  M  A  T

     GGGGTTTCACAGCAGACAATGGACCTCCTTAATATCATCAGCCAGTTCCTTTTGTTCTTT   1637
521  G  V  S  Q  Q  T  M  D  L  L  N  I  I  S  Q  F  L  L  F  F

     AAGTCCTGTGTCACCCCAGTCCTCCTTTTCTGTCTCTGCAAACCCTTCAGTCGGGCCTTC   1697
541  K  S  C  V  T  P  V  L  L  F  C  L  C  K  P  F  S  R  A  F

     ATGGAGTGCTGCTGCTGTTGCTGTGAGGAATGCATTCAGAAGTCTTCAACGGTGACCAGT   1757
561  M  E  C  C  C  C  C  C  E  E  C  I  Q  K  S  S  T  V  T  S

     GATGACAATGACAACGAGTACACCACGGAACTCGAACTCTCGCCTTTCAGTACCATACGC   1817
581  D  D  N  D  N  E  Y  T  T  E  L  E  L  S  P  F  S  T  I  R

     CGTGAAATGTCCACTTTTGCTTCTGTCGGAACTCATTGCTGAAGGAC                1864
601  R  E  M  S  T  F  A  S  V  G  T  H  C  *
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Fig. 1. Nucleotide sequence and deduce amino acid sequence for GPCR/CNS. Numbers to the left refer to the first amino acids on the lines and the numbers to the right refer to the last nucleotides on the lines. The TGA stop codon is denoted by an asterisk.

identified that separately contained the 5′ and 3′ regions of the GPCR/CNS open-reading frame. These clones were sequenced to determine intron–exon borders. One intron was identified at position 1040 of the cDNA sequence. Long range PCR analysis using exonic primers flanking the intron border estimated the intron size at 21 kb (data not shown). Sequence analysis of the ends of the PCR product confirmed its identity. Genomic sequence within the single intron that flanks the two exons is shown in Fig. 2. The P1 clone sequences confirmed our cDNA sequence.

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GCCCTATATAGAGgtaatgccttccaggggctctcaagctagtggctttatctgttttcg
ggattatagcatcagagaactgctgctggggatgcacctatctagctgaacgctttcttt
tatctgggg.................~21 Kb.........................
ctttttatgcttacaatgattcagctgacgtcctgagcccagttacccatgcctcattct
ggaaaactgttctctctctctctctcattttttatttgtccatattttttttttacagtt
ttgccaatctgcatttatttcctagtgctttgatctttcgggggacaccgggaataaatg
tcttctatgttcccccacagGTCGCTTCTCTGGGAG
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Fig. 2. Intron–Exon border mapping of the GPCR/CNS. Exon sequence is shown in upper-case letters and intron sequences are shown in lower-case letters. A ct repeat is in boldface type.

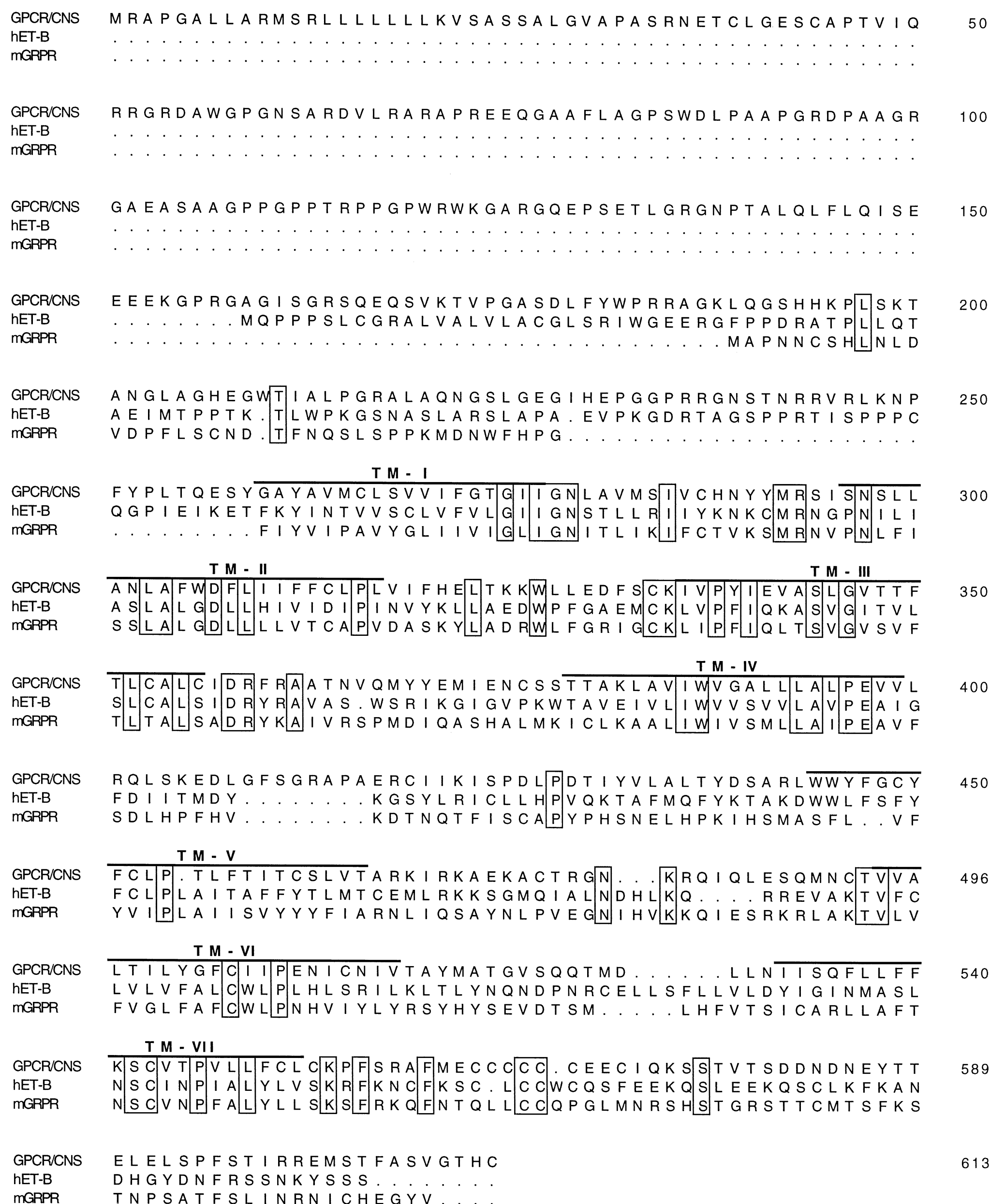

Fig. 3. Sequence identity between the predicted GPCR/CNS amino acid sequence and two members of the G protein-coupled receptor family. The aligned sequences are human GPCR/CNS, human ET-B receptor and murine GRP receptor. Numbers to the right refer to the last GPCR/CNS amino acid in the numbered lines. Columns that are boxed indicate identical residues at that position. Gaps represented by dots were inserted to maximize the alignment. Transmembrane domains are noted by a dark solid line.

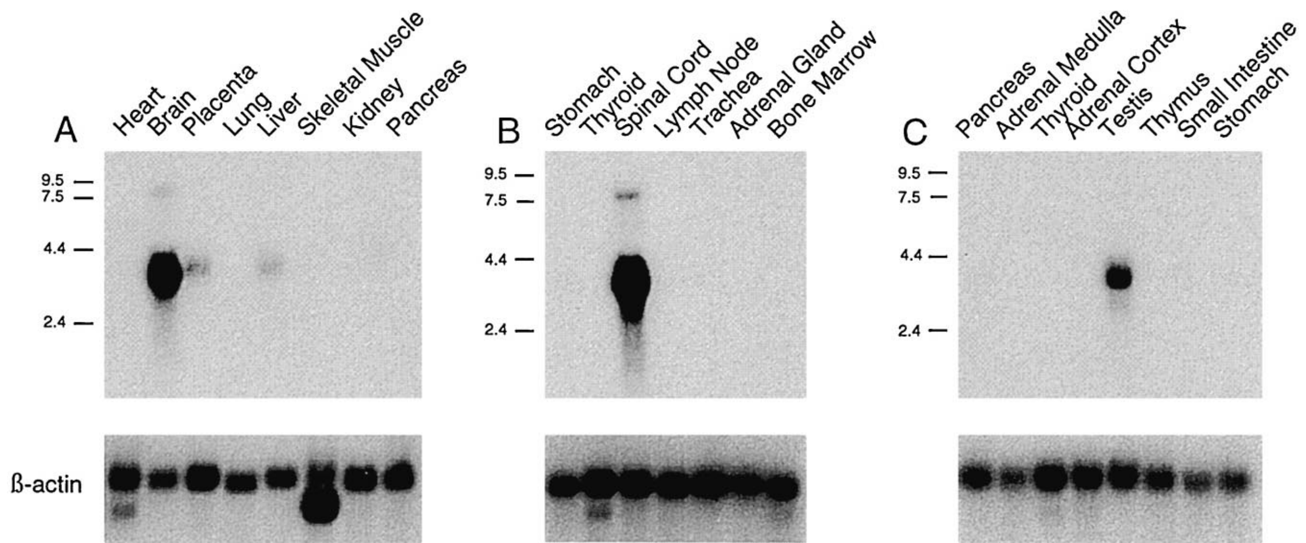

Fig. 4. GPCR/CNS mRNA expression levels in various human tissues. Human tissue Poly A^+ RNA blots obtained from Clontech were analyzed by RNA gel blot hybridization. The upper panels represent blots hybridized to the GPCR/CNS cDNA probe and the numbers to the left indicate RNA size markers (in kb). Lower panels represent the respective blot rehybridized to a β-actin cDNA probe to verify equal loading of intact mRNA in all lanes of the gel.

3.2. Amino acid sequence comparisons

A computer search of protein databases revealed that the deduced amino acid sequence has high overall sequence identity to an orphan GPCR (~98%) recently described [16] and ~23% overall homology to both the murine GRP-R and hET-B receptor, members of the G protein-coupled receptor superfamily. An alignment of GPCR/CNS, mGRP-R and hET-B receptor is shown in Fig. 3. Many of the conserved amino acids appear to lie within transmembrane domains.

3.3. GPCR/CNS mRNA expression levels in human tissues

We used Poly A^+ RNA Northern blot hybridization analysis to examine the tissue distribution of GPCR/CNS mRNA. A single GPCR/CNS transcript of ~3.7 kb is expressed at high levels in brain and spinal cord, whereas intermediate levels were detected in testes and low level expression observed in liver and placenta (Fig. 4, panels ABC). To further localize GPCR/CNS expression in the brain, the GPCR/CNS probe was hybridized to poly A^+

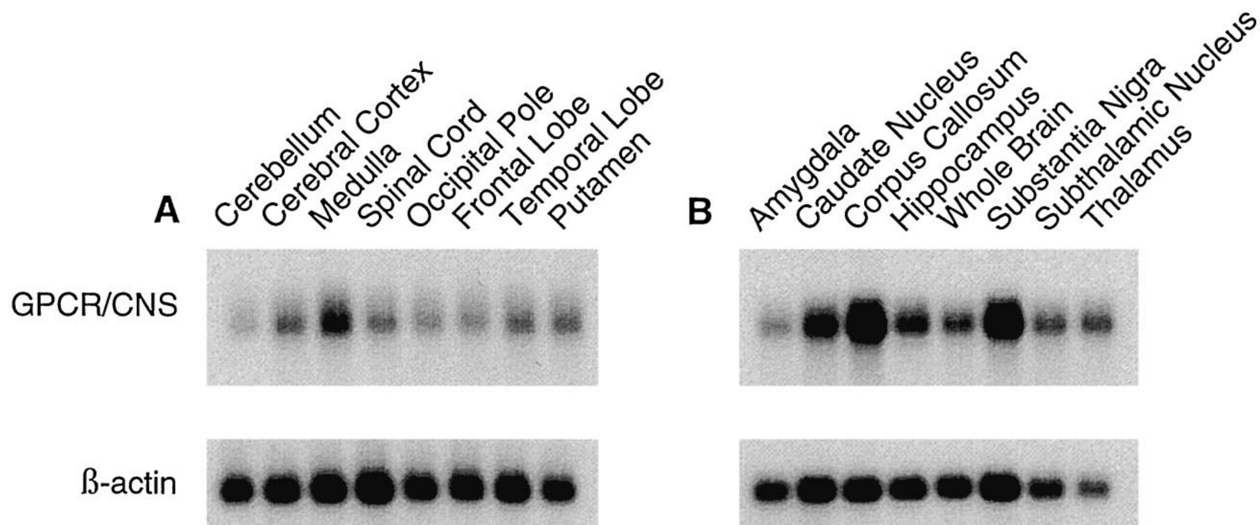

Fig. 5. GPCR/CNS mRNA expression levels in human brain tissues. Human brain tissue Poly A^+ RNA blots obtained from Clontech were analyzed by RNA gel blot hybridization. The upper panels represent blots hybridized to the GPCR/CNS cDNA probe and only the region of the autoradiogram that contained a GPCR/CNS mRNA hybridization signal is shown. Lower panels represent the respective blot rehybridized to a β-actin cDNA probe to verify equal loading of intact mRNA in all lanes of the gel.

Table 1
Ability of ^{125}I-[Tyr^4]Bn or ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) to specifically bind parental BALB/B1 cells, mGRP-R transfected cells and GPCR/CNS transfected cells

	^{125}I-[Tyr^4]Bn binding (cpm)		^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) binding (cpm)	
	Total	+1 μM competitor	Total	+1 μM competitor
Parental BALB/B1 cells	97	79	233	210
mGRP-R transfected cells	7355	310	7557	742
GPCR/CNS transfected cells	221	209	474	355

Unlabelled 1 μM Bn or 1 μM [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) competed ^{125}I-[Tyr^4]Bn or ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) binding, respectively. Each value was determined in duplicate.

RNA derived from a number of human brain regions. All brain regions examined showed expression of GPCR/CNS mRNA, with highest mRNA expression levels noted in corpus callosum and substantia nigra (Fig. 5, panels A and B).

3.4. Ligand binding assays

The ability of GPCR/CNS stably transfected in BALB fibroblasts to specifically bind ^{125}I-Bn or ^{125}I-[DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) was examined. Northern blot analysis of 17 GPCR/CNS stable transfectants was performed in order to identify the clone expressing the highest GPCR/CNS mRNA level and this clone was used in ligand binding and inositol phosphate assays. Recently, [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) was identified as a high affinity agonist for all four Bn receptor subtypes [10]. As shown in Table 1, no specific binding of either radioligand was observed in cells expressing high levels of transfected GPCR/CNS mRNA. In contrast, both tracers bind GRP-R.

3.5. Analysis of agonist-induced inositol trisphosphate elaboration

In radioligand binding experiments such as those performed in this study where the tracer is present at concentrations of about 50 pM, no detectable binding can be measured if the affinity of the receptor for the radioligand is lower than 50 nM (Battey and Sainz, manuscript in preparation). To determine if GPCR/CNS might be a low affinity Bn receptor, we measured agonist-activated inositol trisphosphate elaboration. As shown in Table 2, the GRP-R shows a robust elaboration of inositol trisphosphates after addition of 10 μM Bn or 10 μM [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) peptide. In contrast, no elaboration of inositol trisphosphate over background is observed when transfected fibroblasts expressing GPCR/CNS are examined using the same assay. As a point of reference, transfected fibroblasts expressing BRS-3 receptor, a low affinity Bn receptor ($K_i > 10$ μM for Bn) (Benya et al., manuscript in preparation), show inositol trisphosphate elaboration over background at micromolar concentrations of Bn using the same experimental paradigm used to study transfected fibroblasts expressing GPCR/CNS.

3.6. Analysis of GPCR/CNS expressed in Xenopus oocytes

Oocytes microinjected with in vitro transcript encoding either GPCR/CNS or mGRP-R were tested for their ability to respond to 1 μM or 10 μM Bn. When activated by an agonist, receptor coupling to heterotrimeric GTP-binding proteins of the G_q family will activate phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol trisphosphate, resulting in release of calcium from intracellular stores. Elevation in intracellular calcium concentrations results in the opening of an endogenous calcium-dependant chloride channel and an efflux of chloride when the oocyte is voltage clamped at −60 mV. A total of 10 μm Bn failed to elicit a response from GPCR/CNS (Fig. 6), while trypsin induced a rapid and robust chloride efflux in the oocyte [5] indicating that the oocyte was capable of a response to an appropriate stimuli.

3.7. Chromosomal localization of GPCR/CNS

Fluorescence in situ hybridization of normal lymphocyte metaphase chromosomes was employed to map the

Table 2
Ability of 10 μM Bn or 10 μM [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) to stimulate phosphatidylinositol hydrolysis in parental BALB/B1 cells, mGRP-R transfected cells and GPCR/CNS transfected cells

	No addition	10 μM BN	10 μM [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14)
Parental BALB/B1 cells	2369	2844	3059
mGRP-R transfected cells	3058	16814	19212
GPCR/CNS transfected cells	3008	2872	2566

Data is given in cpm and each value was determined in duplicate.

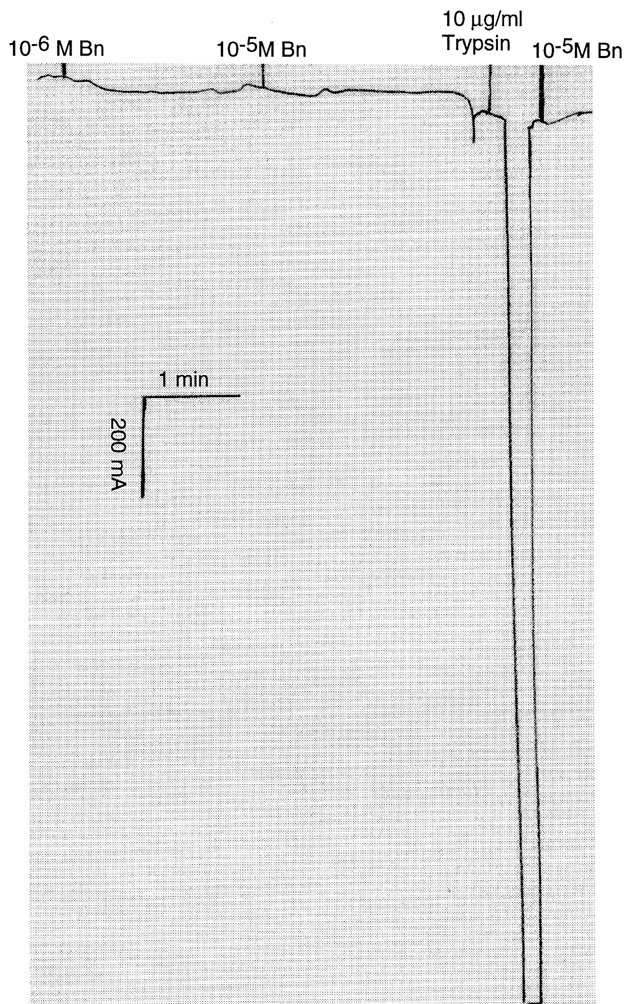

Fig. 6. Ability of Bn or trypsin to induce chloride currents in oocytes expressing GPCR/CNS. Trace of 10^{-6} M Bn, 10^{-5} M Bn and 10 μg/μl trypsin-mediated responses.

chromosomal position of GPCR/CNS. A total of 80 metaphase chromosomes were analyzed with 65 exhibiting specific labelling. The GPCR/CNS gene was mapped to 7q31 (data not shown).

4. Discussion

The mammalian Bn-like GPCR family currently consists of the GRP-R, NMB-R and BRS-3, which share amino acid sequence identity of approximately 50%–60%. Here we further characterize a recently described gene by Zeng et al. [16] that encodes a putative G protein-coupled receptor. Zeng et al. proposed this gene belongs to the ET-B-like receptor family. Our analysis of the deduced GPCR/CNS protein sequence indicates equivalent sequence identity (~ 23) to Bn receptor subtypes and ET-B receptor and may represent a GPCR that belongs to no known receptor family. The expected protein sequence differs from that previously reported [16] in 12 positions and our cDNA sequence was confirmed by sequencing the human genomic P1 clones. These differences are scattered throughout the open reading frame and probably are attributed to sequencing errors. Notably, our sequence contains a conserved alanine in TM-II and a conserved cysteine in TM-VI relative to ET-B and GRP-R whereas the previously reported sequence contains a valine and glycine, respectfully (Fig. 3). The deduced GPCR/CNS protein contains a longer amino terminal domain preceding the first transmembrane domain when compared to the four known Bn receptors. Longer amino terminal domains are common among GPCRs that bind larger ligands as is the case for the luteinizing hormone receptor. This observation suggests that perhaps the endogenous high affinity ligand for GPCR/CNS is a larger molecule than the Bn-like peptides.

Our analysis of the genomic structure of GPCR/CNS revealed a single intron approximately 21 kb in length was identified within the open reading frame. This genomic organization differs from the three mammalian Bn receptors (GRP-R, NMB-R and BRS-3 receptor), where the coding region is interrupted by two introns positioned at identical locations in all three coding regions. This lack of similarity in genomic organization between known Bn receptors and GPCR/CNS is consistent with the lower amino acid sequence identity observed when GPCR/CNS is compared to the known Bn receptor sequences. Both observations suggest that GPCR/CNS is a more distant relative of the four known Bn receptors. Partial sequencing of the single intron in GPCR/CNS revealed a C-T base repeat (Fig. 2) that may be useful as a marker in chromosomal studies if it proves to be highly polymorphic in length.

We assayed the ability of GPCR/CNS-transfected fibroblasts to bind either [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) or Bn. The newly identified agonist [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) has a greater affinity (up to one order of magnitude) for known Bn receptor subtypes compared to previously known ligands (i.e., Bn). In our studies, we find no evidence that either [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6–14) or Bn can bind to GPCR/CNS. When GPCR/CNS mRNA is expressed in transfected fibroblasts, we fail to observe any specific binding of Bn radioligands that are known to bind all four known Bn receptors with high affinity. In addition, we fail to observe any evidence of coupling to G proteins from the G_q subfamily even at agonist concentrations in excess of those required to activate the known Bn receptors. These results could be explained by a failure of the GPCR/CNS protein to fold properly, or insert in the plasma membrane correctly in the heterologous Balb/c fibroblast host.

To examine the possibility that BALB fibroblasts are not an appropriate host for GPCR/CNS expression, we expressed GPCR/CNS in *Xenopus* oocytes, a host cell known to express many functional neuropeptide and neurotransmitter GPCRs. No evidence for Bn-stimulated eleva-

tion in intracellular calcium was observed in oocytes injected with an in vitro transcript encoding GPCR/CNS. The most economical explanation for these data is that GPCR/CNS is not a Bn receptor and the ligand for this receptor remains to be determined. This interpretation of the data is supported by the fact that GPCR/CNS shows much lower amino acid identity to the GRP-R than any other member of the Bn receptor subfamily and the three exon genomic architecture preserved among the genes for mammalian Bn receptors GRP-R, NMB-R and BRS-3 is not preserved in GPCR/CNS. However, we cannot rule out the possibility that GPCR/CNS failed to fold properly, or insert into the plasma membrane correctly, in both the heterologous expression systems employed in our studies. In this case, we would have observed a failure to bind ligands, or activate second messenger effectors, even if GPCR/CNS were a member of the Bn receptor subfamily of GPCRs. In addition, Zeng et al. [16] were unable to demonstrate specific binding to radiolabelled endothelin-1 or -3 in transfected cells, nor did endothelin-1 or -3 induce intracellular Ca^{+2} mobilization or cAMP production in cells expressing this putative receptor.

Consistent with the results by Zeng et al. [16], Northern blot analysis detected high GPCR/CNS mRNA levels in human brain. In addition to this important finding, we show high GPCR/CNS mRNA levels in spinal cord, forming the basis for naming this receptor GPCR/CNS. Additional Northern blot analysis of poly A^+ RNA obtained from various brain regions revealed detectable expression in all regions with somewhat higher expression in the corpus callosum and substantia nigra. These data suggest that GPCR/CNS may have a unique functional role in the central nervous system. More information about the role of this receptor in CNS function awaits the discovery of a naturally occurring agonist.

Note added in proof

While this article was in press, a human cDNA sequence corresponding to GPCR/CNS was published by D. Marazziti *et al.* Genomics **45** (1997) 68–77 and named GPR37 in accordance with the Genome Nomenclature Committee.

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