Research report

Distribution of the GABA$_B$ receptor subunit gb2 in rat CNS

Janet A. Clark [a],[*],[1], Éva Mezey [b],[2], Alan S. Lam [a], Tom I. Bonner [a]

[a] *Laboratory of Genetics, National Institute of Mental Health, 36 Convent Drive, MSC 4094, Bethesda, MD 20892-4094, USA*
[b] *Basic Neuroscience Program, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892-4094, USA*

Abstract

We have identified and isolated human and rat cDNAs for a novel receptor, gb2, with 38% homology to the GABA$_B$ receptors gb1a and gb1b. These receptors comprise a new subfamily of seven transmembrane G protein-coupled receptors (GPCRs) that share structure and sequence similarities with the metabotropic glutamate receptors. In situ hybridization histochemistry using an antisense probe to this novel receptor mRNA shows a distribution in rat CNS nearly identical to that for the gb1 receptor, although some regions showed significant differences. Specifically, message levels for gb2 were virtually absent in the caudate/putamen, and significantly lower in the medial basal hypothalamus, septum and brainstem as compared with gb1 message levels. In contrast to gb1, gb2 mRNA was never detected in white matter suggesting that gb2 message is found exclusively in neurons. Finally, in rat brain regions showing significant overlap of message for gb1 and gb2, the transcripts are often found in the same cells. Data from our previous work showing that coexpression of gb2 with gb1 is necessary for expression of a functional receptor together with the detailed anatomical data presented here indicate that native GABA$_B$ receptors function as heteromeric proteins, the most abundant form being the gb1/gb2 receptor. However, the more limited distribution of gb2 receptor mRNA suggests that there are brain regions where GABA$_B$ receptors are composed of gb1 and as yet unidentified family members. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

GABA$_B$ receptors, originally identified as a distinct subclass of GABA receptors by Bowery et al. [3,4,11] are well established as members of the G protein-coupled receptor (GPCR) super family. They mediate a variety of inhibitory cellular processes through modulation of ion channels and adenylyl cyclase activity [6,17,22]. Kaupmann et al. [14] have described the first primary sequence for a GABA$_B$ receptor which was isolated from rat brain using expression cloning techniques. The rat GABA$_B$ receptors identified, rgb1a and rgb1b, are alternatively spliced variants of the same gene product which differ in their amino terminal sequence and are most closely related to metabotropic glutamate receptors. They exhibit a reduced affinity for GABA$_B$ receptor compounds as compared with native receptors yet the rank order of potency for these compounds is similar to that described in the literature for GABA$_B$ receptors in tissue preparations. Recent work by Couve et al. [10] has shown that recombinant gb1 receptors expressed in heterologous systems do not reach the cell surface and are trapped in the endoplasmic reticulum. These data suggest that the gb1 receptors require additional information for functional targeting to the plasma membrane that appears to be missing in the cells used to study receptor function thus far. Until recently there had been no meaningful study of the function of these receptors. We and others have recently reported that coexpression of gb1a or gb1b with a related protein, gb2, in various heterologous expression systems results in the expression of functional GABA$_B$ receptors [12,15,19,23,29]. The results of these studies suggest that the native GABA$_B$ receptor may be a heterodimer of gb1 and gb2 subunits.

Here we describe the isolation of human and rat cDNAs for the gb2 GABA$_B$ receptor subunit and the CNS distribution of gb2 mRNA in the rat. This is only the second GABA$_B$ receptor primary sequence to be identified. In situ hybridization histochemistry revealed that gb2 and gb1 mRNA exhibit overlapping expression patterns in many rat

* Corresponding author. Fax: +1-301-435-5465; e-mail: janet@codon.nih.gov
[1] Co-first authors.
[2] Co-first authors.

brain regions with transcripts for both receptors found in the same cells in a vast majority of cases. The colocalization data are supportive of our previous studies showing that coexpression of gb2 with gb1 in heterologous expression systems is necessary to observe $GABA_B$ receptor function [23]. In addition, rgb2 mRNA exhibits a more discrete expression pattern in some rat brain regions than rgb1 and appears to be absent from white matter suggesting that it is predominantly localized to neurons. If functional $GABA_B$ receptors are composed of heteromers then the finding that rgb1 message is abundant in many brain regions and in white matter where rgb2 is barely detectable suggests that there may be additional, as yet unidentified $GABA_B$ receptors.

2. Materials and methods

2.1. Cloning of hgb2

A tblastn search of the Genbank EST database using the amino acid sequence of the rat gb1 receptor as query detected three ESTs, R76139, R80448, and Z43654, which showed distant homology to transmembrane domains 5–7 and another EST, T07261, which showed similarity to transmembrane domains 1–3. Primers designed from these sequences (JC136 gcgggatccCTTCGGCACGAAT-ACCAGGCAGAGGGT for 5′ extension; JC137 ccg-gaattcCAGAAGCTCATAAAGATGTCGAGTCC and JC135 ccggaattcTTCTTAGCTTTGGGAGACCCGCA-ACGT for 3′ extension) were used with the Clontech AP1 primer (Clontech; Palo Alto, CA) to do 5′ and 3′ RACE using Clontech Marathon-Ready human brain cDNA as template and touchdown PCR conditions (94°C, 30 s and 72°C, 4 min for five cycles; 94°C, 30 s and 70°C, 4 min for five cycles; 94°C, 30 s and 68°C, 4 min for 20 cycles). JC135 and JC136 primers were derived from the transmembrane domains 5–7 ESTs while JC137 was derived from T07621. JC136 and JC137 primers were used together to generate a product of 722 bases which when sequenced confirmed that the ESTs were derived from the same mRNA. 3′ products from RACE with JC135 and JC137 were reamplified with the Clontech AP2 primer yielding two products of 1.2 and 1.7 kb in size but lacking a poly(A) signal. A tblastn search of the Genbank EST database with the 3′ sequences detected an EST, AA323988, which overlapped with the query sequence and extended the sequence through an A-rich region. gb2–15 (AGCACTAGAACTCCAGCTGGAAGTCA) was designed based on EST AA323988 and used in a 3′ RACE reaction as described above to yield a 2.3-kb band that when sequenced was shown to include a poly(A) signal. 5′ RACE with JC136 yielded a 0.88-kb NotI/BamHI fragment which when sequenced showed weak amino acid homology to rgb1. JC153 (GCCTACGATGGCATCTGG-GTCATCGC) was designed based on the JC136 5′ RACE

product sequence and paired with JC136 to yield a 1.1-kb fragment. Further 5′ extensions were obtained by using Clontech Advantage-GC PCR (1 μl GC-melt per 50 μl reaction, PCR conditions: 94°C, 15 s and 68°C, 3.5 min for 30 cycles) and nested primers JC152 (CAGATTCCAGC-CTTGGAGGGACTCTGC) followed by JC186 (TGCATCGTAGAAGGCTTTCAACCCTTTTGC) and adapter primers AP1 followed by AP2.

pcDhgb2I was generated by subcloning a NaeI/XhoI fragment from a JC153/JC165 (JC165: AATAAGGCTC-GAGGTCAGGTGCCAA) 2.2 kb PCR product and an EcoRI/NaeI fragment from a JC171/JC154 (JC171: CAGCAGCCCGCCGCTCTCCATCAT; JC154: gcgcg-taatacgactcactatagggGTAGTTGAAGTCCTGGATCCG) 1.0 kb PCR product into EcoRI/XhoI digested pcDNA1/amp (Invitrogen; Carlsbad, CA). The expression construct pcDhgb2II was constructed by subcloning a NotI/XmnI 5′ RACE fragment obtained with AP2/JC186 into a NotI/XmnI digested pcDhgb2I. pcD3.1hgb2 was made by subcloning an EcoRI/XbaI fragment from pcD-hgb2II into EcoRI/XbaI digested pcDNA3.1 (Invitrogen).

The JC135 and JC137 3′ RACE reactions yielded a closely spaced pair of bands. When individual clones were sequenced they appeared to represent alternatively spliced 3′ ends of the hgb2 cDNA (Genbank accession numbers AF056085, AF095723 and AF095724). The 1.7-kb JC137 clone (hgb2 (c), Fig. 2) lacked an exon corresponding to bases 3239–3319. The JC153/JC165 2.2 kb clone (hgb2 (b), Fig. 2) that was used to make the expression construct pcDhgb2II lacked an exon corresponding to bases 3161–3238. The relative intensities of the PCR bands indicated that the two smaller gb2 receptor variants combined are several-fold less abundant than the longer form.

2.2. Cloning of rgb2

rgb2 (Genbank accession AF058795) was obtained by screening pools of clones from a rat cortex cDNA library made in the Okayama–Berg vector pCD.1 [2] using Southern blot analyses. DNA was prepared from each of 49 pools representing an average of 5×10^5 clones per pool and digested with XhoI to release the inserts from the vector DNA. Five microgram of XhoI digested pool DNA was run on a 0.8% agarose gel and transferred to a nitrocellulose supported membrane (BA-S 85, Schleicher & Schuell; Keene, NH) using a TurboBlotter (Schleicher & Schuell). Nitrocellulose membranes were hybridized with a ^{32}P-labeled random primed (Boerhinger Mannheim; Indianapolis, IN) hgb2 JC137/JC136 PCR fragment in $3 \times$ Denhardt's and $3 \times$ SSC at 60°C. Hybridization was followed by two 15-min washes in $3 \times$ SSC at 60°C. Blots were imaged on the Fujix PhosphorImaging system (Fuji; Japan). Bacteria from two pools were each used to make 22 subpools. DNA was prepared from these pools and analyzed as above. Two positive pools (10I and 37O) of

3000 colonies each were screened by colony hybridization following standard protocols [28]. Two similar clones were isolated from this screen the longest of which (5200 kb clone 37094a) was sequenced and found to lack a start site. RACE was performed using the high GC conditions described above with nested primers JC190 (CGCTG-GTGCAGGGATCATTGGAGAA) and JC191 (CATAT-TCTGGTCAAACTGGCCAAGG) paired with AP1 followed by AP2 using Clontech Marathon-Ready rat brain cDNA as template to yield the 5' end of the rgb2 receptor. A *Xho*I/*Bgl*II fragment from the 5' RACE of rgb2 and a *Bgl*II fragment from 37O94a were subcloned into *Xho*I/*Bam*HI digested pBSSKII(−). Restriction enzyme mapping with *Xho*I and *Bam*HI established the correct orientation of the *Bgl*II fragment. pCD3.1rgb2 was made by subcloning a *Kpn*I/*Bam*HI fragment of the pBSSKII(−) plasmid with a *Bam*HI/*Xba*I fragment from 37O94a into *Kpn*I/*Xba*I digested pcDNA3.1.

2.3. Cloning of rgb1b and rgb1a

rgb1b was obtained by amplification of Clontech rat brain Marathon-Ready cDNA with JC140 (ccggaattcccac-cATGGGCCCGGGGGGACCCTGTACC) and JC141 CTAGTCTAGATCACTTGTAAAGCAAATGTACTCGA-T). pBSrgb1b was made by ligation of an *Eco*RI/*Xba*I fragment of rgb1b into *Eco*RI/*Xba*I prepared pBSSKII (−). pcDrgb1b was constructed by subcloning an *Eco*RI/*Not*I fragment from pBSrgb1b into *Eco*RI/*Not*I prepared pcDNA1/amp (Invitrogen). rgb1a 5' sequence was obtained by 5' RACE with AP1 or 2 paired with JC144 (GAGTAGTTCGTACAAGTACTTGGTGGC) using Clontech rat brain Marathon-Ready cDNA as template. A rgb1a 5' fragment for vector construction was generated by amplification of the JC144 RACE product with JC139 (ccggaattcccaccATGCTGCTGCTGCTGCTGGTGC-CTCTC) and JC144. pBSrgb1a was made by ligation of an *Eco*RI/*Hpa*I fragment from the JC139/JC144 PCR product into *Eco*RI/*Hpa*I digested pBSrgb1b. pcDrgb1a was constructed by subcloning an *Eco*RI/*Bst*EII fragment from the pBSrgb1a into *Eco*RI/*Bst*EII digested pcDrgb1b. An *Eco*RI/*Not*I fragment from pcDrgb1a was subcloned into pcDNA3.1 (Invitrogen) to make pcD3.1rgb1a.

2.4. Northern analysis

Clontech multiple human tissue northerns were hybridized with the 0.88 kb *Not*I/*Bam*HI fragment (bases 732–1613 of hgb2) at 42°C in 5 × SSPE, 10 × Denhardt's, 50% formamide, 2% SDS, 100 μg/ml denatured salmon sperm DNA. Following hybridization blots were washed three times for 10 min each in 0.2 × SSPE, 1% SDS at 60°C. Blots were imaged on the Fujix PhosphorImaging system (Fuji).

2.5. In situ hybridization histochemistry

Preparation of rat brain sections and probes as well as hybridization of rat brain slices were performed as described previously [7,18] (http://intramural.nimh. nih.gov/lcmr/snge/Protocol.html). Sections were hybridized with ^{35}S-UTP-labeled antisense and sense riboprobes directed against rgb2 or rgb1 mRNA. Probes were generated by amplification of rgb2 with JC216 paired with JC217 or with JC218 paired with JC219 (JC216: cgcgcaat-taaccctcactaaaggACAACAGCAAACGTTCAGGC; JC217: gcgcgtaatacgactcactatagggCATGCCTATGATGGTGAG; JC218: cgcgcaattaaccctcactaaaggCTGAGGACAAAC CCTGACGC; JC219: gcgcgtaatacgactcactatagggGAT-GTCTTCTATGGGGTC) or by amplification of rgb1 with JC160 paired with JC161 (JC160:cgcgcaattaaccctcactaaag-gAAGCTTATCCACCACGAC; JC161:gcgcgtaatacgactcac tatagggAGCTGGATCCGAGAAGAA) and labeled as described previously.

2.6. Simultaneous detection of gb2 and gb1 transcripts by in situ hybridization histochemistry

For simultaneous detection of gb1 and gb2 transcripts procedures were the same as described above except that rgb1 probes were labeled with digoxigenin-UTP. Brain slices were simultaneously hybridized with probes to both gb1 and gb2 receptors and digoxigenin-labeled gb1 receptor message was detected using a peroxidase-conjugated antibody amplification scheme involving biotinyl tyramide (TSA amplification procedure, NEN; Boston, MA) and subsequent detection with streptavidin-conjugated CY3. Detection of the radiolabeled rgb2 probe was performed after detection of the digoxigenin-labeled rgb1 probe on the same brain slices.

3. Results

3.1. hgb2 and rgb2 amino acid sequences

Through EST database searches, polymerase chain reaction, and library screening as described in Section 2 we have identified and isolated human and rat homologues of a novel receptor, hgb2 and rgb2, with homology to the GABA$_B$ receptors, rgb1a and rgb1b, identified by Kaupmann et al. [14]. Fig. 1 shows a schematic of the hgb2 and rgb2 receptor nucleotide sequences denoting the placement of oligonucleotide primers and restriction enzyme sites used to generate each of these constructs and the expression plasmids. hgb2 receptor fragments were amplified from human brain cDNA while rgb2 was isolated from a rat cortex cDNA library followed by amplification of rat brain cDNA to obtain the 5' end of the receptor including

```
                                      R76139
                           T07261    R80448              AA323988
               NotI    XmnI   NaeI       Z43654    XhoI
  hgb2    |_____|_____|_____█████████████████_____|_____
                  →         →    →    →       ←
                JC171     JC153 JC137 JC135   JC165
                   ←          ←         ←          →
                 JC186      JC154     JC136      gb2-15
                    →
                  JC152

               BglII BamHI BglII              XbaI
  rgb2    |_____|___|____|____████████████____|_____
                    ←
                  JC190
                    ←
                  JC191
```
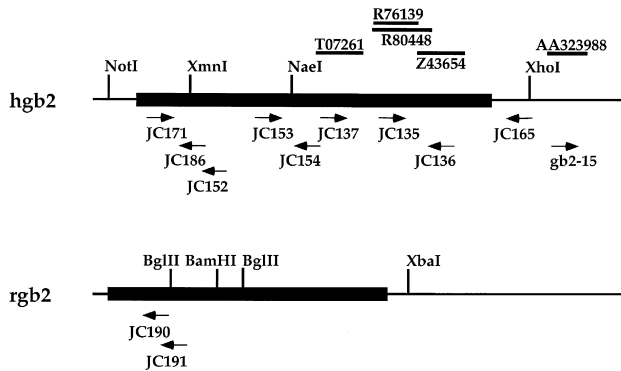
Fig. 1. Schematic diagrams of hgb2, and rgb2 sequences showing the relative location of ESTs, oligonucleotide primers and restriction enzyme sites used in constructing the cDNAs. Constructs were generated as described in Section 2. Thin bars represent noncoding regions of the cDNAs; thick bars represent the coding regions. Left facing arrows represent sense primers; right facing arrows represent antisense primers.

the start site. The resulting 5786 base pair sequence generated for hgb2 includes an open reading frame of 2826 bases. Some of the ESTs corresponding to hgb2 had previously been mapped by radiation hybrid analysis to the D9S287–D9S176 region of chromosome 9q (http://www.ncbi.nlm.nih.gov/genemap98/map.cgi?MAP = G3-&BIN = 323&MARK = SHGC-32035). The combined 5′ RACE product and insert for rgb2 spans 5459 base pairs and includes an open reading frame of 2823 bases. The cDNA sequences for hgb2 and rgb2 predict proteins of 942 and 941 amino acids, respectively, with relative molecular mass of 104 kD.

Sequence similarity and hydropathy profiles [20] of the predicted proteins demonstrate that they share structural features with the gb1 receptors [14], the parathyroid cell calcium-sensing receptor, and the family of metabotropic glutamate receptors [26]. Significant features in the hgb2 and rgb2 sequences include seven putative transmembrane regions, a large extracellular amino terminus of 476 residues, and four potential sites for N-linked glycosylation (Fig. 2). Potential signal peptide cleavage sites were identified in the gb2 receptor sequences (Fig. 2) using a neural network method for identification of signal peptides and prediction of their cleavage sites [9,24] (http://www.cbs.dtu.dk/services/SignalP/). Comparison of the amino acid sequences for hgb2 and rgb2 with that for

Fig. 2. Alignment of human gb2, rat gb2, rat gb1a and rat gb1b receptor amino acid sequences. C-terminal alternative splice variants of human gb2 are designated a, b, and c. Dots indicate residues identical in all receptors. Hydrophobic domains are denoted by bars and labeled with the transmembrane domain (TM) number. Proposed signal peptide cleavage sites [24] (http://www.cbs.dtu.dk/services/SignalP/) for human and rat gb2 are denoted with an X. Potential N-linked glycosylation sites are designated by an asterisk over the appropriate residues.

```
hgb2    MASPRSSGQPGPPPPPPPPPPPARLLLLLLLPLLL-------------------
rgb2    MASPPSSGQPR-PPPPPPPPPARLLLLPLLLSLLL-------------------
rgb1a             MLLLLLVPLFLRPLGAGGAQTPNATSEGCQIIHPPWEGGI
rgb1b             MGPGGPCTPVGWPLPLLLVMAAGVAPVWA----------------

hgb2    -----------------------------------------------------
rgb2    -----------------------------------------------------
rgb1a   RYRGLTRDQVKAINFLPVDYEIEYVCRGEREVVGPKVRKCLANGSWTDMDTPS
rgb1b   -----------------------------------------------------

hgb2    -----------------------------------------------------
rgb2    -----------------------------------------------------
rgb1a   RCVRICSKSYLTLENGKVFLTGGDLPALDGARVEFRCDPDFHLVGSSRSVCSQ
rgb1b   ---------------------------------------------------S-
                                       X
hgb2    PLAPG-AWGWARGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAI
rgb2    WLAPG-AWGWTRGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAI
rgb1a   GQWSTP-KPHCQVNRTPHSERRAVYIGALFPMS-GGWPG--GQACQPAVEMAL
rgb1b   HS-PHLPRPHPRVPPHPSSERRAVYIGALFPMS-GGWPG--GQACQPAVEMAL
                     •                 •      •••    •••• •
hgb2    EQIRN-ESLLRPYFLDLRLYDTECDNAKGLKAFYDAIKYGPNHLMVFGGVCPS
rgb2    EQIRN-ESLLRPYFLDLRLYDTECDNAKGLKAFYDAIKYGPNHLMVFGGVCPS
rgb1    EDVNSRRDILPDYELKLIHHDSKCDPGQATKYLYELLYNDPIKIILMPG-CSS
         •          •   •              • •  ••    •   ••
hgb2    VTSIIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLL
rgb2    VTSIIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLL
rgb1    VSTLVAEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLF
         • •••   •• ••• •••      •• •••• ••   ••
hgb2    KHYQWKRVGTLTQDVQRFSEVRNDLTGVLYGEDIEISDTESFSNDPCTSVKKL
rgb2    KHFRWRRVGTLTQDCQRFSEVRNDLTGVLYGEDIEISDTESFSNDPCTSVKKL
rgb1    EKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNL
         •  • ••    •    ••   •••• •       •••   •• •••
hgb2    KGNDVRIILGQFDQNMAAKVFCCAYEENMYGSKYQWIIPGWYELSWWEQVHTE
rgb2    KGNDVRIILGQFDQNMAAKVFCCAFEESMFGSKYQWIIPGWYEPAWWEQVHVE
rgb1    KRQDARIIVGLFYETEARKVFCEVKRERLFGKKYVWFLIGWYADNWFK---TY
         • •   •••• •   •••  ••••  •    ••• •• •••
                    *
hgb2    ANSSRCLRKNLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQY-EREYNN-KRS
rgb2    ANSSRCLRRSLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQF-EREYNS-KRS
rgb1    DPSINCTVEEMTEAVEGHITTEIVMLNPANTRSISNMTSQEFVEKLTKRLKRH
         • •          • ••• •     •••     •   ••      ••
hgb2    GVGPSKFH-G-YAYDGIWVIAKTLQRAMETLHASSRHQRIQDFNYTDHTLGRI
rgb2    GVGPSKFH-G-YAYDGIWVIAKTLQRAMETLHASSRHQRIQDFNYTDHTLGRI
rgb1    PEETGGFQEAPLAYDAIWALALALNKTSGGGGRSGV--RLEDFNYNNQTITDQ
         ••        •••  ••         •• • •    • •••••
                *
hgb2    ILNAMNETNFFGVTGQVVFR-NGERMGTIKFTQFQDSREVKVGEYNAVADTLE
rgb2    ILNAMNETNFFGVTGQVVFR-NGERMGTIKFTQFQDSREVKVGEYNAVADTLE
rgb1    IYRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQLQGGSYKKIGYYDSTKDDL-
         •  ••• •  •• • •••   • • •  •  ••   •  •     ••
                *                          TM1
hgb2    IINDTIRFQGSEPPKDKTIILEQLRKISLPLYSILSALTILGMIMASAFLFFN
rgb2    IINDTIRFQGSEPPKDKTIILEQLRKISLPLYSILSALTILGMIMASAFLFFN
rgb1    SWSKTDKWIGGSPPADQTLVIKTFRFLSQKLFISVSVLSSLGIVLAVVCLSFN
         • ••    •••  •• ••••   •  •••    •    ••• •• •    ••
                        TM2                          TM3
hgb2    IKNRNQKLIKMSSPYMNNLIILGGMLSYASIFLFGLDGSFVSEKTFETLCTVR
rgb2    IKNRNQKLIKMSSPYMNNLIILGGMLSYASIFLFGLDGSFVSEKTFETLCTVR
rgb1    IYNSHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDGYHIGRSQFPFVCQAR
         • • •       • • •••  • •• ••   •• •••••       ••• •
                                              TM4
hgb2    TWILTVGYTTAFGAMFAKTWRVHAIF--KNVKM-KKKIIKDQKLLVIVGGMLL
rgb2    TWILTVGYTTAFGAMFAKTWRVHAIF--KNVKM-KKKIIKDQKLLVIVGGMLL
rgb1    LWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKEWRKTLEPWKLYATVGLLVG
         • •   •   • • •••• •  •      •   •    • ••  • •
                          TM5
hgb2    IDLCILICWQAVDPLRRTVEKYSMEPDPAGRDISIRPLLEHCENTHMTIWLGI
rgb2    IDLCILICWQAVDPLRRTVERYSMEPDPAGRDISIRPLLEHCENTHMTIWLGI
rgb1    MDVLTLAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSSKKMNTWLGI
         •  • •  •  •••• ••     •    •  • •  • •••••       ••••
                                  TM6
hgb2    VYAYKGLLMLFGCFLAWETRNVSIPALNDSKYIGMSVYNVGIMCIIGAAVSFL
rgb2    VYAYKGLLMLFGCFLAWETRNVSIPALNDSKYIGMSVYNVGIMCIIGAAVSFL
rgb1    FYGYKGLLLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMI
         •• ••••••  • ••• •• ••••    ••    •• •••• • •  • • •
           TM7
hgb2    TRDQPNVQFCIVALVIIFCSTITLCLVFVPKLITL--RTNPDAATQN-RRF-Q
rgb2    TRDQPNVQFCIVALVIIFCSTITLCLVFVPKLITL--RTNPDAATQN-RRF-Q
rgb1    LSSQQDAAFAFASLAIVFSSYITLVVLFVPKMRRLITRGEWQSETQDTMKTGS
         •        • • •• •••  •••   ••••••
hgb2    FTQNQKKEDSK-TSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKDLEEVT
rgb2    FTQNQKKEDSK-TSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKDLEEVT
rgb1    STNNNEEEKSRLLEKENRELEKIIAEKEERV-SELRHQLQSRQQLRSRRHPPT
         • •      •    •    •     •     •
hgb2    MQLQDTPEKTTYIKQNHYQELNDILNLGNFTESTDGGKAILKNHLDQNPQLQW
rgb2    MQLQDTPEKTTYIKQNHYQELNDILSLGNFTESTDGGKAILKNHLDQNPQLQW
rgb1    PPDPSGGLPRGPSEPPDRLSCDGSRVHLLYK


hgb2(a)   NTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILHHAYLPSIGGVDASCVS
hgb2(b)   NTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILHH---------------
hgb2(c)   NTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILHHAYLPSIGGVDASCVS
rgb2      NTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILHHAYLPSIGGVDASCVS


hgb2(a)   PCVSPTASPRHRHVPPSFRVMVSGL
hgb2(b)   -----------RHVPPSFRVMVSGL
hgb2(c)   PCVSPTASPRHRTTLGRGVCCRNTVGSGCGEAGHHGWPLRTTRMALRWTG
rgb2      PCVSPTASPRHRHVPPSFRVMVSGL


hgb2(c)   RGRGRLGT
```

rgb1a and rgb1b (Fig. 2) shows 28% amino acid identity which rises to 40% when conservative amino acid substitutions are taken into account. Homology between the gb2 and gb1 receptors is not limited to the hydrophobic regions but instead spans the entire protein. As was the case with gb1, gb2 shares sequence homology with metabotropic glutamate and the calcium-sensing receptors [14].

3.2. rgb2 expression in rat CNS

Analyses of Northern blots of human mRNA from several brain regions and peripheral tissues with a probe directed against the hgb2 receptor reveals a single mRNA species of 6.2 kb (Fig. 3). hgb2 receptor mRNA is expressed in all of the brain areas tested although the level of expression seems to vary significantly with the highest in the cortex and thalamus and the lowest in the corpus callosum, caudate nucleus, and medulla. In addition, a weak 6.2 kb band is detected in spinal cord mRNA. No hgb2 receptor mRNA is detected by Northern analysis in any of the peripheral tissues examined with the exception of heart where a weak band is observed.

In situ hybridization histochemistry was carried out using sense and antisense probes directed against both the rgb2 and rgb1 receptors for comparison as described in Section 2. The rgb1 receptor probe is directed against a region common to both the rgb1a and rgb1b receptors (Section 2), therefore, both rgb1a and rgb1b mRNAs are recognized by this probe. Both rgb2 and rgb1 receptor mRNAs are heterogeneously expressed in the rat brain with many regions showing an overlap for the two receptor transcripts.

Gb2 and gb1 receptor mRNAs are equally abundant in the cortex, thalamus, medial and lateral geniculate bodies, habenula, and cerebellum (Fig. 4). Unlike gb1 receptor message, which is very abundant and heterogeneously expressed throughout the rat CNS [14], the distribution of the gb2 receptor message is somewhat more discrete. There are several brain regions that show major differences in the levels of mRNA for the two receptors. Gb2 receptor message is virtually absent from the caudate/putamen (Figs. 4B–D and 5C) and its levels are significantly lower than that of the gb1 receptor mRNA in the septum, the preoptic area and the hypothalamus (Figs. 4C–F and 5B–E). Whereas gb1 receptor message is homogeneously expressed throughout the regions in which it is found, this is not always the case for gb2 receptor message. Despite the relatively low levels of gb2 receptor mRNA in the hypothalamus, gb2 message is abundantly expressed in the magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei (Fig. 4F and Fig. 5E) and expressed at medium to high levels in the dorsomedial hypothalamic nucleus and the suprachiasmatic nuclei (Fig. 4E, and Fig. 5E). Gb2 receptor message is expressed at relatively low levels in catecholaminergic brain areas such as the substantia nigra and locus coeruleus (Fig. 4H and J, respectively), areas where gb1 receptor message is abundant (data not shown). Message for both receptors are detected in high levels in the hippocampus, however, a significant difference in the expression patterns for these two receptor mRNAs in the hippocampus was observed. Gb2 receptor message shows a graded pattern of expression in the hippocampus with relatively low levels in the CA1 area, somewhat higher levels in CA2, and very high levels in CA3 and the dentate gyrus (Fig. 4G). Gb1 receptor message is abundantly and homogeneously ex-
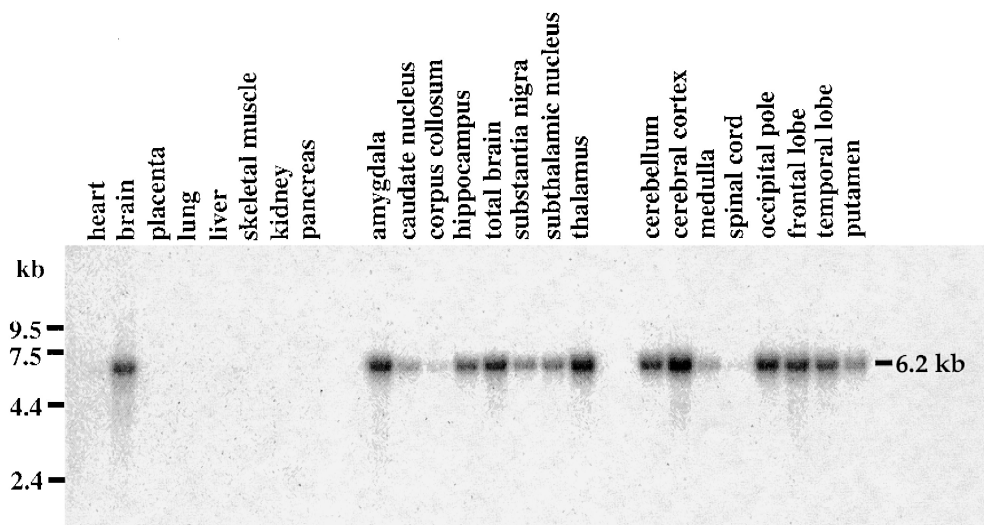

Fig. 3. hgb2 mRNA expression in human CNS and peripheral tissues as determined by Northern blot analysis. Clontech multiple human tissue Northerns were hybridized with a probe directed against the hgb2 receptor as described in Section 2. Note the weak band present in heart and spinal cord human mRNA.
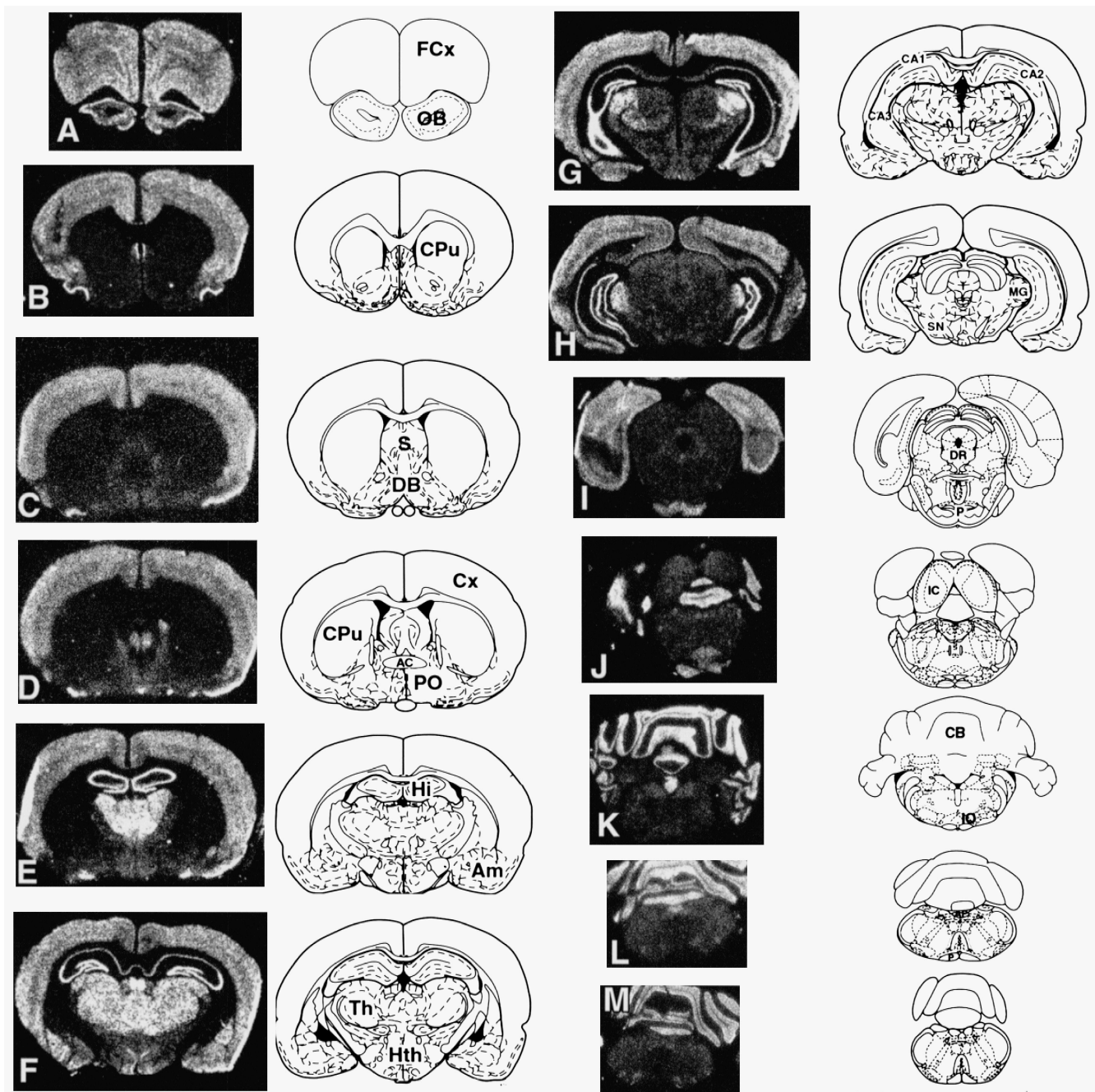
Fig. 4. Distribution of the gb2 receptor mRNA in coronal sections of rat brain by in situ hybridization histochemistry. (A–M) Autoradiographic images were scanned from X-ray film opposed to the microscope slides after hybridization and exposed for 5 days. Scanned images were saved in Adobe Photoshop 5 and combined with drawings from Paxinos and Watson's Atlas [25] showing the corresponding levels. Abbreviations: AC = accumben nucleus; Am = amygdala; AP = area postrema; CA1,CA2,CA3 = hippocampal subdivisions; CB = cerebellum; Cpu = caudate/putamen; Cx = cortex; DB = diagonal band of Broca; DR = dorsal raphe; FCx = frontal cortex; Hi = hippocampus; Hth = hypothalamus; IC = inferior colliculus; IO = inferior olive; MG = medial geniculate nucleus; OB = olfactory bulb; P = pons; PO = preoptic hypothalamus; S = septum; SN = substantia nigra; Th = thalamus.

pressed throughout all areas of the hippocampus (data not shown).

In the brainstem, gb2 receptor mRNA is expressed at equally high levels in the cerebellum (Purkinje and granule cells) and in a few restricted nuclei including the inferior olive, lateral reticular formation, solitary tract nucleus, and the dorsal parabrachial nucleus (Fig. 4J–M). However, gb1

receptor message was detected at high levels in all brainstem nuclei (data not shown).

In contrast to gb1 receptor mRNA, we did not detect hybridization to gb2 receptor mRNA in white matter, suggesting that expression of gb2 receptor message is restricted to neurons in rat brain. Our detailed examination of the rat CNS has revealed that gb1 mRNA is found in
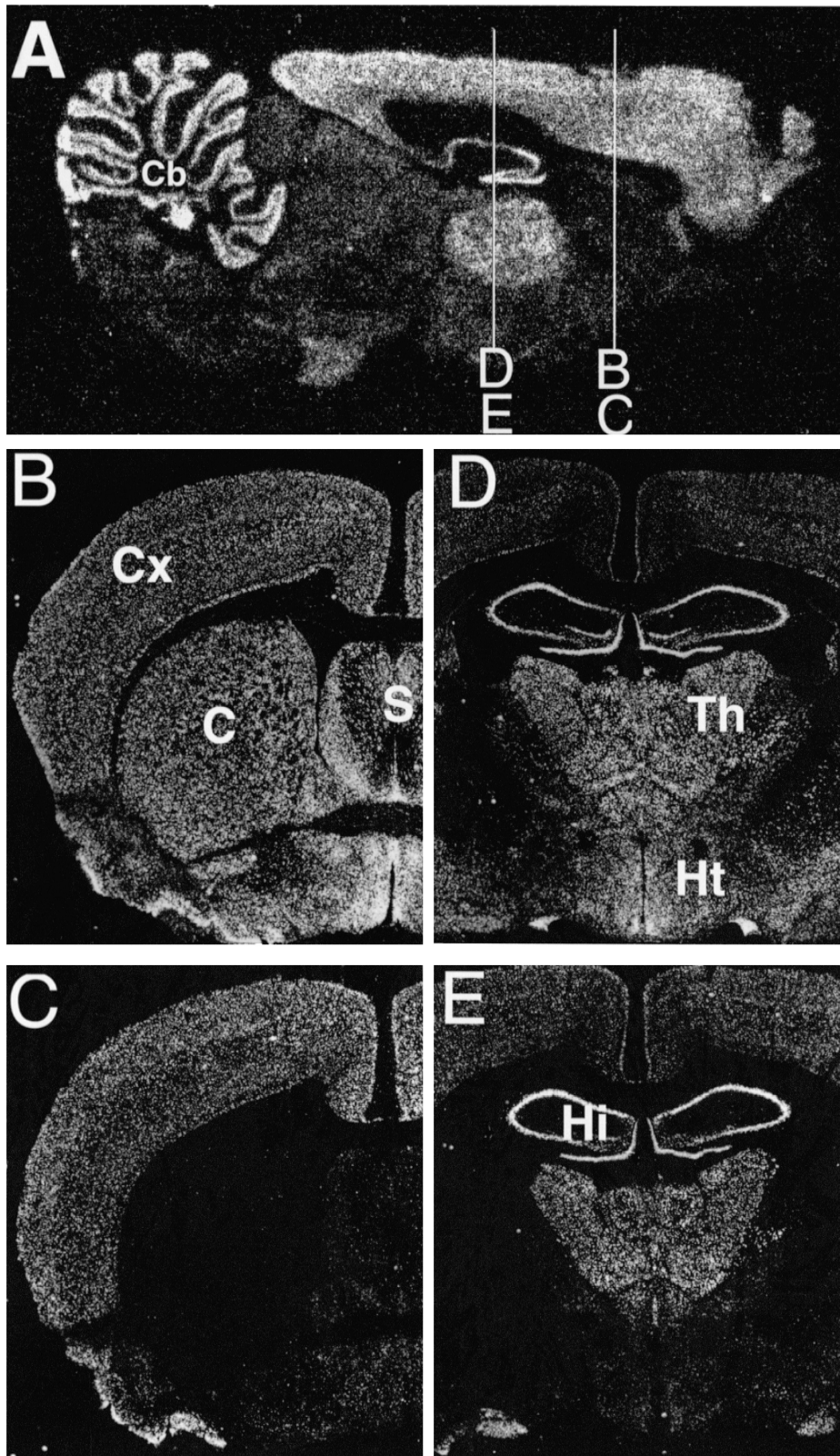
Fig. 5. Comparison of the distribution of gb2 and gb1 receptor mRNA in the rat brain by in situ hybridization histochemistry. (A) shows an autoradiographic image of a parasagittal rat brain section hybridized with the rat gb2 probe. The perpendicular lines depict the levels at which 12 μm thin coronal sections (B–E) are shown. (B) and (C), and (D) and (E) show the same levels (adjacent sections) hybridized with rat gb1 (B and D) and gb2 (C and E) receptor probes. Note the lack of grains over the caudate nucleus, septum and preoptic area in (C) as compared with (B), and the lack of grains over the hypothalamus in (E) as compared with (D). Abbreviations: C = caudate nucleus; Cx = cerebral cortex; Hi = hippocampus; Hth = hypothalamus; PO = preoptic area; S = septum; Th = thalamus. Scale bar = 1 mm.
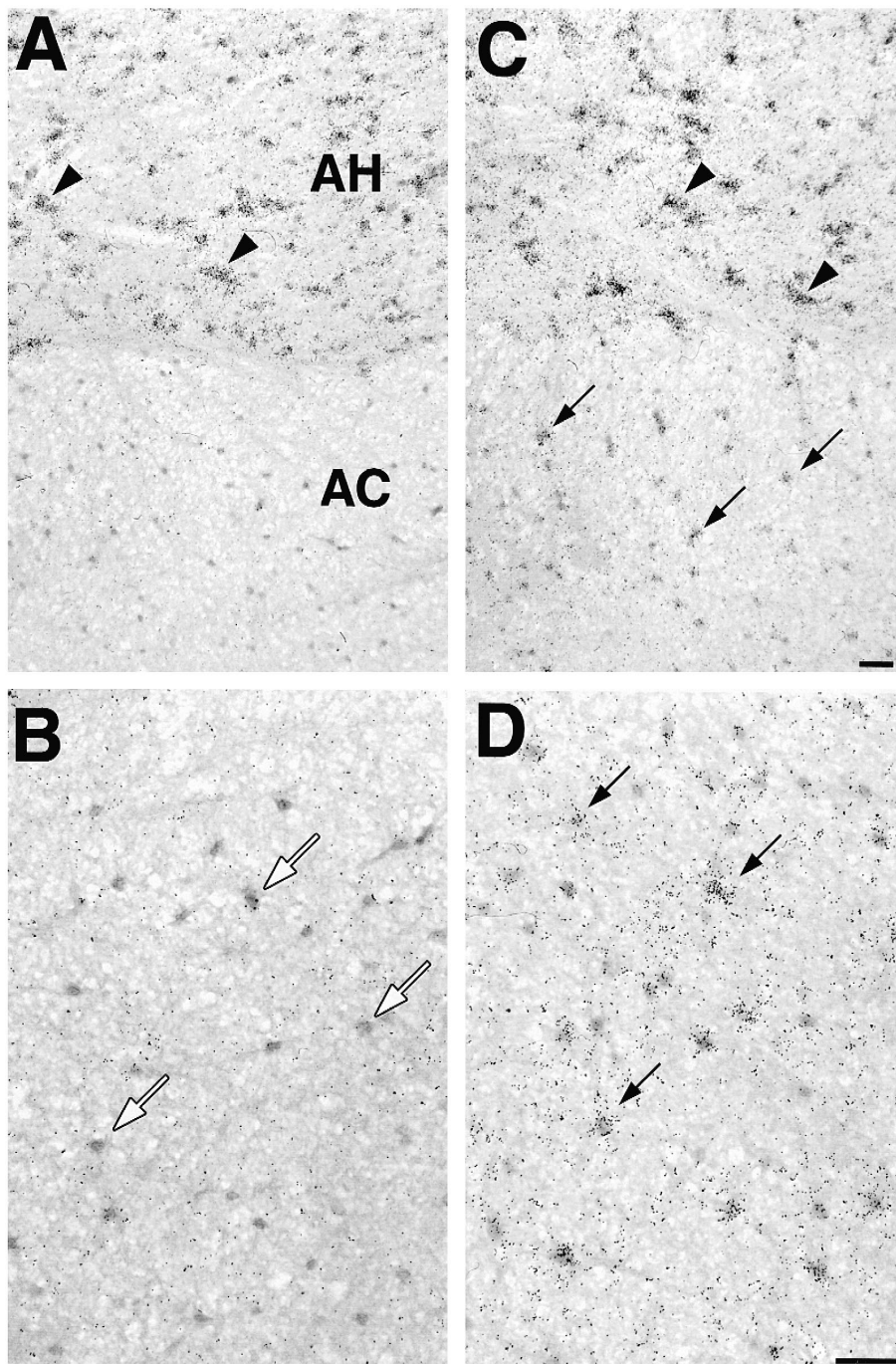
Fig. 6. Localization of gb2 and gb1 receptor mRNA in rat spinal cord. Gb2 (A and B) and gb1 (C and D) receptor mRNA in the anterior horn and anterior column of the spinal cord. Note specific hybridization of gb2 and gb1 probes to motorneurons in the anterior horn (A and C) and specific hybridization of the gb1 probe to glial cells in the anterior column at a higher magnification (D). Arrowheads denote neurons specifically hybridizing with the probes; white arrows denote nuclei of glial cells that do not hybridize with the gb2 probe; black arrows denote glia that specifically hybridize with the gb1 probe. AC = anterior column; AH = anterior horn. Scale bar = 25 μm.

Fig. 7. Colocalization of mRNA for $GABA_B$ receptor subtypes by in situ hybridization histochemistry in rat brain. Rat gb1 probe was digoxigenin-UTP labeled and developed using anti-digoxigenin HRP, the TSA amplification method and CY3-tyramide. Rat gb2 probe was labeled using ^{35}S-UTP. The slides were emulsion-coated and autoradiography was developed after 4 weeks. (A–C) Cerebellum, (D–F) hippocampus, (G–I) cortex, and (J–L) vestibular ganglion. (A), (D), (G) and (J) show autoradiography with the gb2 receptor probe. Red cells in (B), (E), (H) and (K) express gb1 receptor mRNA in the same fields as shown in (A), (D), (G) and (J). (C), (F), (I) and (L) are overlays of images (A) and (B), (D) and (E), (G) and (H), and (J) and (K), respectively. Arrows denote some of the double-labeled cells. Note the fibers of the eighth nerve passing through the middle of the image in (K).
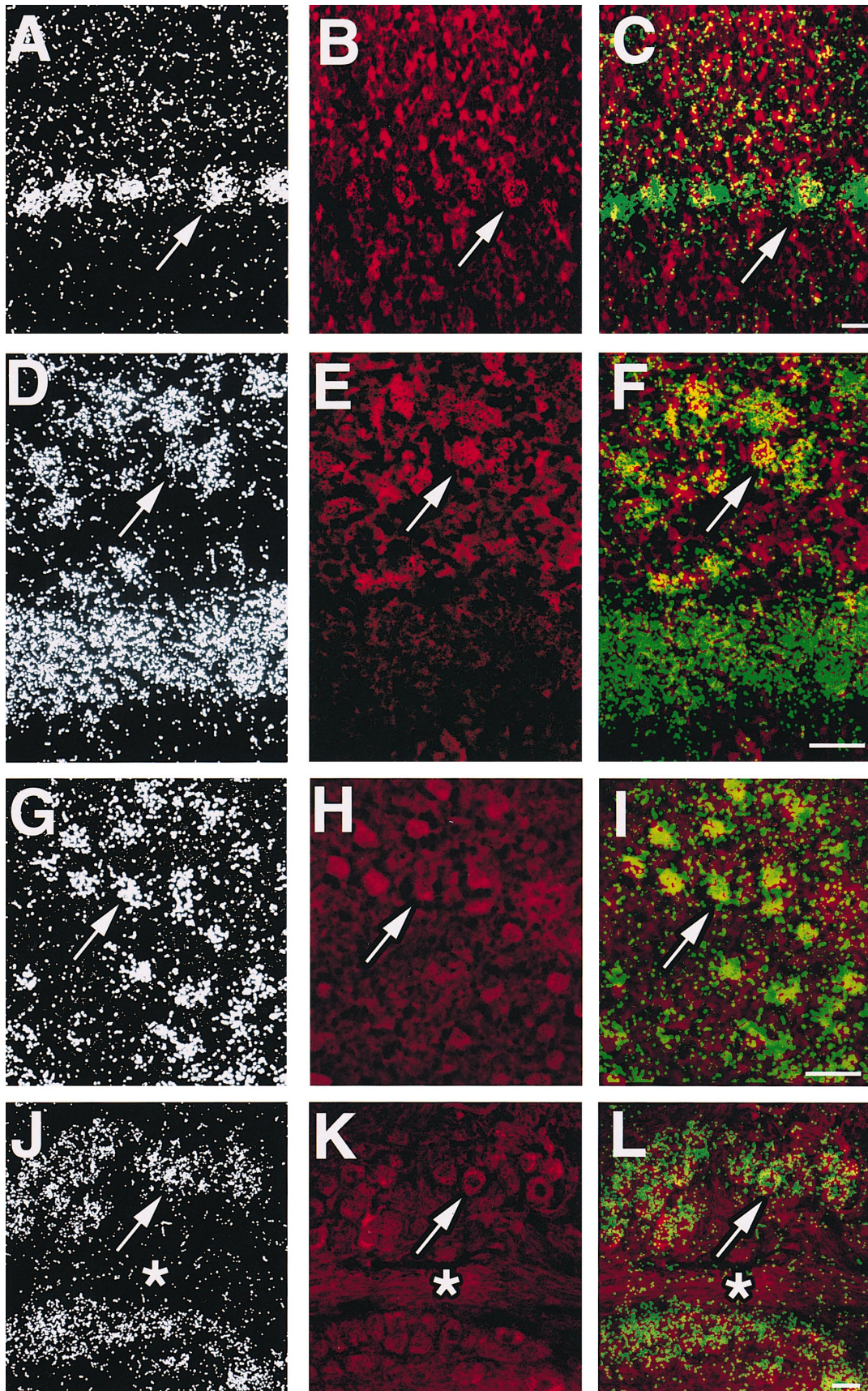
glia in white matter throughout the CNS including the corpus callosum (Fig. 5B). In the spinal cord gb1 and gb2 transcripts are abundant in neurons of the gray matter but only the gb1 probe hybridizes specifically to glial cells in the white matter (Fig. 6). Gb1 receptor message was detected in glial cells throughout the brain in addition to glial cells in white matter (data not shown). While we have been unable to detect hybridization to rgb2 mRNA in glia under the conditions used, more detailed studies of rgb2 mRNA expression and protein localization at the cellular level are necessary before we can rule out this possibility.

Since many brain regions showed overlapping expression patterns for gb2 and gb1 receptor message, colocalization studies for the two receptors were performed to determine if message for the two receptors is found in the same cells in these regions. Fig. 7 shows colocalization of gb1 and gb2 receptor message in the cerebellum (Fig. 7A–C), hippocampus (Fig. 7D–F), cortex (Fig. 7G–I) and vestibular ganglion (Fig. 7J–L), four of the many rat brain regions examined. Neurons in the circumventricular organs including organum vasculosum laminae terminalis (OVLT), subfornical organ, median eminence, subcommissural organ, and area postrema express both receptor mRNAs at equally high levels (data not shown). In addition, the ventricular ependyma of the third ventricle in the hypothalamus expresses high levels of both receptor mRNAs (data not shown). In all other major brain regions tested where both mRNAs were found, the gb2 and gb1 receptor mRNA is colocalized with $\geq 95\%$ of gb2 expressing cells also expressing gb1.

4. Discussion

There are several clinical situations in which $GABA_B$ receptors may prove to be potential sites for therapeutic intervention. Disrupted GABAergic neurotransmission has been implicated in a number of neurological and psychiatric disorders, including epilepsy, schizophrenia, and affective disorders. Due to the potential therapeutic value of $GABA_B$ receptors, it is important to identify the molecular structures of $GABA_B$ receptors and understand how they function. Recent work by Kaupmann et al. [14] revealed the first primary sequence information for members of this receptor family. Binding experiments with membranes expressing the cloned gb1 receptor clearly showed a rank order of potency for $GABA_B$ receptor-specific compounds in accordance with those observed for native $GABA_B$ receptors. However, attempts to show consistent and robust $GABA_B$ receptor function in any in vitro system tested were unsuccessful [14,16] most likely due to inappropriate plasma membrane targeting [10]. In this manuscript we describe the identification and isolation of human and rat homologues of a novel receptor, gb2, with 35% amino acid sequence homology to the gb1 receptors identified by Kaupmann et al. [14]. Like the gb1 receptors,

we were unable to detect gb2 receptor function in the various assays tested, yet unlike the gb1 receptors, no detectable binding could be observed with $GABA_B$ receptor agonists or antagonists in membranes expressing the cloned gb2 receptor. However, we and others have shown that coexpression of gb1 and gb2 receptors together in many different in vitro expression systems yields functional $GABA_B$ receptors [12,15,19,23,29]. In addition, we have shown that coexpression of gb1 and gb2 receptors results in the formation of heterodimers that are thought to represent the functional form of a $GABA_B$ receptor.

In performing a detailed analysis of the CNS distribution of gb2 receptor message in rat brain by in situ hybridization histochemistry we observed that there are many regions where gb1 and gb2 receptor message overlap including the cortex, hippocampus, thalamus and many hindbrain areas including the cerebellum and ganglia. As shown in Fig. 7, at the cellular level, the vast majority of cells expressing gb2 receptor message in the rat brain also express gb1 receptor message. Kaupmann et al. [15] have gone a step further in Purkinje cells only demonstrating that both gb1 and gb2 receptor message and proteins colocalize. These data do not prove that native $GABA_B$ receptors in these cells are gb1/gb2 heterodimers, yet these findings strongly suggest that native $GABA_B$ receptor function in these cells involves both gb1 and gb2 receptors.

In contrast to the robust and ubiquitous expression of gb1 receptor mRNAs in rat brain, gb2 receptor message is somewhat more discretely localized. Levels of gb2 receptor message in caudate/putamen, hypothalamus, septum, preoptic area, and substantia nigra are low to undetectable (Figs. 4 and 5), whereas gb1 receptor message is present at moderate to high levels. Likewise, gb2 receptor message is undetectable in glial cells of white matter throughout the rat brain and spinal cord. Expression of gb1 receptor message, however, is detected in glial cells of all white matter and in glia throughout many areas of the brain. These data indicate that gb2 message is found exclusively in neurons.

The previously described binding of GABA to rat brain in the presence of the $GABA_A$ receptor agonist isoguvacine [5,8] parallels the observed distribution of gb2 receptor message presented in Figs. 4 and 5. GABA binding in regions including cortex, thalamus, and cerebellum is relatively robust as is expression of both gb1 and gb2 receptor message. Certain regions including the septal area, caudate/putamen, hypothalamus and brainstem, with the exception of the interpeduncular nucleus exhibited relatively low levels of GABA binding [5,8]. In this study we have shown that these same regions that exhibit poor GABA binding express low to undetectable levels of gb2 receptor message. Regions where robust GABA binding overlap with robust expression of both gb1 and gb2 receptor message most likely represent regions where cells are coexpressing a functional $GABA_B$ receptor in the form of

a gb1/gb2 heterodimer. Agreement of the gb1/gb2 message expression levels with the levels of GABA binding in rat brain lends further support to the hypothesis that functional native GABA$_B$ receptors in many major brain regions are composed of gb1/gb2 heteromers. However, gb1/gb2 receptor heteromers may not account for GABA$_B$ receptor function in regions where gb1 receptor message is expressed with little or no detectable gb2 receptor message. The composition of functional GABA$_B$ receptors in these regions remains to be determined.

There are several possible reasons why one may find functional GABA$_B$ receptors in regions where expression of gb1 receptor message is robust and gb2 receptor message is relatively low. Cells exhibiting low levels of gb2 receptor message may express the appropriate cellular components necessary for the expression of functional homomultimeric gb1 receptors at the cell surface. Evidence for the existence of homomultimeric receptors comes from recent reports which have shown that GPCRs, including the parathyroid calcium-sensing and metabotropic glutamate receptors, form homomultimeric structures which may be important for function [1,27]. At least two groups have shown that expression of gb1 using various cell culture systems results in accumulation of a major portion of the expressed protein in intracellular compartments [10,29]. Coexpression of gb1 with gb2 results in the targeting of gb1 to the cell surface in the form of a functional gb1/gb2 heteromer [29]. In addition, binding assays done with membranes from cells expressing gb1/gb2 heteromers result in a 10- to 100-fold shift to the left in the apparent affinities for agonists when compared with assays done with membranes expressing gb1 only [15,29]. These data suggest that the formation of a homomultimeric gb1 receptor and targeting of such a receptor to the cell surface may involve accessory proteins that are not expressed by the cells used for in vitro study of this receptor. Recent work has shown that accessory proteins termed receptor-activity modifying proteins (RAMPs) have a dramatic affect on the cell surface expression and pharmacology of the GPCR calcitonin-receptor-like receptor [21]. Therefore, it is possible that similar proteins are involved with the expression and targeting of functional GABA$_B$ receptors in cells expressing gb1 receptor message alone. Another recently identified family of proteins, RGS or regulator of G protein signaling molecules, have been shown to modify metabotropic glutamate receptor-mediated ion channel modulation [13]. Therefore, it is possible that our inability to observe gb1 receptor function in in vitro systems is due to the presence or absence of accessory proteins such as RGS-like proteins.

Alternatively, regions where only gb1 mRNA is detected may express heteromeric GABA$_B$ receptors composed of gb1 and other as yet unidentified GABA$_B$ receptor subunits. Homology-based cloning strategies should reveal additional members of this GABA$_B$ receptor family if they exist. Given the extent and diversity of most receptor families, it would not be surprising to find additional GABA$_B$ receptor subunits.

Finally, gb2 receptor mRNA levels may be undetectable in particular brain regions due to highly specific gene regulation, whereby gb2 receptor message is only expressed after a particular cellular insult. In such a case, functional GABA$_B$ receptor heteromers would only be expressed when a cell needs the protection afforded by GABA$_B$ receptor activation.

While it is now well established that gb1/gb2 heterodimers form in in vitro systems and exhibit the properties expected of a GABA$_B$ receptor, it is still unclear if these heteromers form in vivo and to what extent. This study provides the first extensive examination of the distribution of gb2 receptor message in the rat CNS. The significant overlap in gb1 and gb2 receptor message expression observed in the same cells in many brain regions suggests that gb1/gb2 heterodimers account for the structure of functional GABA$_B$ receptors throughout much of the brain. However, detailed immunohistochemical analyses are needed to confirm the existence of gb1/gb2 heterodimers in vivo. Furthermore, much work needs to be done to determine the composition and functional properties of GABA$_B$ receptors in cells where gb1 receptor message is exclusively expressed.

Acknowledgements

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