

# Cloning, pharmacological characteristics and expression pattern of the rat GABA<sub>A</sub> receptor $\alpha_4$ subunit

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A cDNA of rat brain encoding the GABA<sub>A</sub> receptor  $\alpha_4$  subunit has been cloned. Recombinant receptors composed of  $\alpha_4$ ,  $\beta_2$  and  $\gamma_2$  subunits bind with high affinity the GABA agonist [<sup>3</sup>H]muscimol and the benzodiazepine 'alcohol antagonist' [<sup>3</sup>H]Ro 15-4513, but fail to bind benzodiazepine agonists. The  $\alpha_4$  subunit is expressed mainly in the thalamus, as assessed by in situ hybridization histochemistry, and may participate in a major population of thalamic GABA<sub>A</sub> receptors. The  $\alpha_4$  mRNA is found at lower levels in cortex and caudate putamen, and is rare in cerebellum.

GABA<sub>A</sub>; Benzodiazepine receptor; In situ hybridization; Thalamus;  $\alpha$  Subunit heterogeneity

## 1. INTRODUCTION

GABA<sub>A</sub> receptors mediate the fast synaptic inhibitory effects of the neurotransmitter GABA in brain. This receptor is a ligand-gated anion channel, and is the target of action for a variety of psychoactive compounds such as barbiturates, benzodiazepines, neurosteroids and ethanol [1,2]. The GABA<sub>A</sub> receptor is assumed to be a pentameric structure composed of subunits belonging to subunit classes  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\rho$  [3,4]. So far, in the rodent five  $\alpha$  subunit types have been identified by cDNA screening. These are  $\alpha_1$  [5],  $\alpha_2$  [6],  $\alpha_3$  [7],  $\alpha_5$  [5,7,8] and  $\alpha_6$  [9,10]. The subunit termed by us and others as  $\alpha_5$  [7,8] has also been termed as  $\alpha_4$  [5]. A cDNA encoding a bovine  $\alpha_4$  subunit has been isolated although not pharmacologically characterized [11], with the existence of a rat  $\alpha_4$  homologue remaining uncertain [11]. The  $\alpha$  subunits are a major factor determining pharmacological diversity in GABA<sub>A</sub> receptors, with different  $\alpha$  subunits combining with a  $\beta/\gamma$  pair to exhibit a broad spectrum of pharmacology [3,8,9,12]. Since it is important to analyze the whole repertoire of GABA<sub>A</sub> receptor subunits in an accessible experimental model, we have cloned the rat  $\alpha_4$  subunit mRNA and studied the sites of its expression in the rodent brain. At the same time, we present the first pharmacological characterization of this subunit.

## 2. MATERIALS AND METHODS

### 2.1. Isolation of cDNA clones

A rat brain cDNA library constructed in  $\lambda$ -zap (Stratagene) was screened using a <sup>32</sup>P-labelled DNA fragment of the bovine  $\alpha_4$  cDNA [11]. A cloned 3.6 kb cDNA was identified and subcloned by in vivo excision into Bluescript plasmid. From this cDNA, a 0.6 kb *Kpn*I fragment, a 0.6 kb *Eco*RI fragment and a 2.2 kb *Eco*RI/*Xba*I fragment were subcloned into M13 vectors [13] and sequenced [14]. The 2.2 kb *Eco*RI/*Xba*I fragment was sequenced with the aid of two internal primers, 5'-TTCTCAAGTTTGCTTCTGG-3' ( $\alpha_{4,1}$ ), and 5'-TGTG-TACCACATATCCCT-3' ( $\alpha_{4,2}$ ). A 2.9 kb *Xba*I fragment containing the entire coding sequence as well as 0.2 kb of 5'-untranslated and 0.8 kb of 3'-untranslated regions was used to construct a eukaryotic expression vector [8] for the rat  $\alpha_4$  subunit.

### 2.2. Pharmacology of recombinant GABA<sub>A</sub>-benzodiazepine receptors

Expression vectors for  $\alpha_4$ ,  $\beta_2$  and  $\gamma_2$  cDNAs were transfected in triple combinations into human embryonic kidney (293) cells (ATCC # CRL 1573) as described previously [12]. Binding studies were carried out identically to previous protocols [8,9,12].

### 2.3. In situ hybridization histochemistry

A 45 base antisense oligonucleotide (5'-CAAGTCGCCAGGCA-CAGGACGTGCAGGAGGGCGAGGCTGACCCCG-3') was constructed to a unique part of the  $\alpha_4$  subunit mRNA, complementary to amino acids 15 to 30 of the signal peptide. A 45 base  $\alpha_1$  oligonucleotide was complementary to the region encoding subunit residues 342–356 [5]. These probes were enzymatically labelled with terminal transferase, at a 30:1 molar ratio of [<sup>35</sup>S]dATP (1200 Ci/mmol) to oligonucleotide. In situ hybridization was performed as described previously [15]. In brief, 14  $\mu$ m cryostat sections were hybridized with 3' end-labelled oligonucleotides in 50% formamide/4 × SSC/10% dextran sulphate overnight at 42°C. Sections were washed to a final stringency of 1 × SSC at 60°C, dehydrated and exposed to XAR-5 (Kodak) film. Competition experiments with an excess (50-fold) of unlabelled probe resulted in blank autoradiographs.

## 3. RESULTS AND DISCUSSION

Screening of a rat forebrain cDNA library with a bovine  $\alpha_4$  cDNA probe at high stringency resulted in the

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-91 GGGAGCCACTCTGCCCTCTCCCTCGCACCCCTGCACAGGGCATCTTGAGAGGCTGGAACCTG  
 -31 GAACAGGCTTAAAGTATGGCATGTGCCGAAGATGGTTCTGTCCAGAGGATCTGCCGAT  
 -29 H V S V V Q K V P A I  
 30 CGTGTGTCTCCGGGTCAGCCTCGCCCTCTGCACGCTGTGTGCTGCGCAGCTTGT  
 -19 V L C S G V S L A L L H V L C L A T T C L  
 90 AAACGAATCCCGAGCAGCAATCAAGGACGAGAAATGTGCCGGAATTTACCGG  
 2 N E S P G Q N S K D E K L C F E N F T R  
 150 TATCGTGGACAGTTTGTGGATGGTTAGATGACACAGACTGCGCTCGGATTTGGGGGTCC  
 22 I L D S L L D G T Y D N R L R P G F G G P  
 210 TGTACAGGCTGAAACTGATATATATGTACAGCTTTGGACCCGTTTGTGATTTGA  
 42 V T E V K T D I Y V T S F G P V S D V E  
 270 AATGGAATACAAATGGATGTGTCTTCAGACAGCATGGATTGACAAAGCATGAATA  
 62 H E Y T H D V F R Q T W I D K R L K Y  
 330 TGATGGCCCATTAATCTGAGGTTGAACAAATATGATGGTACCAAAAGTTTGGACCCG  
 92 D C P I E I L R L L N N H M V T R V W T P  
 390 TGATCTTTCTTCAGGAATGGAAGAAATCTGTCTCCCATACATGACAGCTCCAAATAA  
 102 D T F F R N G K K S V S H N M T A P N K  
 450 ACTTTTGAATATGAGAACTGGCACTATTTATACAAATGAGACTCACCATAATGTC  
 122 L F R I H R N G T I L Y T M R L T I S A  
 510 GGATGTCCCATGAGACTGGTGGATTTCTATGACGGTGCATGCTGCCCTTTGAAAT  
 142 E C P H R L V D F P M D G H A C P L K F  
 570 TGGAGTTATGCATATCCAAAGATGAGATGATACACCTGGACCAAGGCCCTGAGAA  
 162 G S Y A Y P K S E M I Y T W T K G P E K  
 630 GTCAGTGGAGTACCAAGAGTCTCGAGCTTAGTCAATGATCTAATTCAGGACGAC  
 182 S V E V P K E S S S L V Q Y D L I G Q T  
 690 TGTATCCAGTACAGATCAATCTATTACAGGTGAATACATCTTATACCGCTGACT  
 202 V S S E T I K S I T G E Y I V M T V Y F  
 750 TCACCTCAGACGGAAGATGGGTATTTTATGATTCAGACATATCCCTGCATCATGAC  
 222 H L R R K M G Y F H I Q T Y I P C I M T  
 810 AGTATCTCTCTCAAGTCTCTGGATCAATAGGAGTCTGCTCCAGCAGAACTG  
 242 V I L S Q V S F W I N K E S V P A R T V  
 870 ATTTCGAATACCAAGCTCTCAGATGACCACTTAAGCATGCTGCGCATCTCTT  
 262 F G I T T V L T M T L S I S A R H S L  
 930 GCCCAAGTCTCTATGCGACTGCCATGAGTGGTTCATAGCTGCTGTTTGTCTGT  
 282 P K V S Y A T A H D W F I A V C E A F V  
 990 ATTTCGGCTCTATGAGTCTGCTGCTCACTATTTCACCAATTCAAATGCAAAA  
 302 F S A L I E F A A V N Y F T N I Q M Q K  
 1050 AGCCAAAGAGATATCAACCTCTCCAGAGTTCAGCTGCCCGAGTACTGAAGGA  
 322 A K K K I S K P P E V P A A P V L R E  
 1110 ANACATACAGAAATCTCTTCAGATACACATGCTAATTTGAACATGAGGAAAGAAC  
 342 K H T E T S L Q N T H A N L N H R K R T  
 1170 AATGCAATAGTCCACTCAGAAATGATGTCAACAGCAGAACTGAGGTGGGAACCATTC  
 362 H A L V H S E S D V N S R T E V G N H S  
 1230 CAGCAAGACACCGCTGCCAGGAGTCTCTGAAACCACTCTCAAGGCCACTTGGCTTC  
 382 K T T A A Q A Q T T E T T P K A H L T  
 1290 CAGTCAAAATCATTGAGCAGGCAATGACGCTGAGACTATCTCTGACAGCAAGAGG  
 402 S P N P F S R A N A A E T I S A A A R G  
 1350 TCTTTGCTGCGCCGATCGCCCTCTCTCAGCGGACCTGAGCCAGCTCTTTGCGGTC  
 422 L S S A A S P S P H G T L Q P A P L R S  
 1410 GCGCTGCTGCGCCGGCATTTGGAGTAGACTTGGCGCATTAAGACAACGTTAATAC  
 442 A S A R P A F G A R L G R I K T T V N T  
 1470 GACAGGGTGCTGGGAATGTGTCAGCAGACCTCTCCCTCTGCTGACCACTCTGCTG  
 462 T G V P G N V S A T P P P S A P P P S G  
 1530 ATCTGCAAGTAAATAGACAAATATGCTCTGATTCTCTTCAGTCAATTTGGGGC  
 482 S G T S K I D K Y A R I L F P V T F G A  
 1590 ATTACATGCTGCTGCTGCTGCTTATTTATTAAGGACCACTGGAGAACATCAGAAAG  
 502 F N M V Y W V Y L S K D T M E K S E S  
 1650 TCTAATGTAATTTTGTGCTAAGCAATTCATAACCGTATGGAATACAGACTGCTT  
 522 L H  
 1710 TTTAAATGTTTTAAAGTAAGTATCTTTACTAAATAAATA 1752

Fig. 1. Nucleotide and deduced amino acid sequence of the rat GABA<sub>A</sub> receptor  $\alpha_4$  subunit. The arrow marks potential cleavage site of signal peptide; N, potential N-glycosylation sites; filled circles, potential protein kinase C phosphorylation sites; open circles, potential cAMP dependent protein kinase phosphorylation site; transmembrane domains are boxed. The dotted line indicates the 15 residue disulfide-bonded loop region.

isolation of a cDNA with an open reading frame of 1656 nucleotides encoding a protein of 552 amino acid residues, including a predicted signal sequence of 19 amino acids (Fig. 1). The deduced amino acid sequence revealed an 88% sequence identity with the bovine  $\alpha_4$  subunit

[5]. Like its bovine counterpart, the rat  $\alpha_4$  polypeptide is predicted to contain a large cytoplasmic portion, making  $\alpha_4$  the largest GABA<sub>A</sub> receptor subunit to date (MW of unglycosylated mature polypeptide, 65 kDa). The putative cytoplasmic sequence contains two potential sites for cAMP dependent phosphorylation and one for protein kinase C phosphorylation (Fig. 1).

The pharmacology of the  $\alpha_4$  subunit was examined by co-expression with  $\beta_2$  and  $\gamma_2$  subunits in cultured mammalian 293 cells (Table I). The  $\alpha_4\beta_2\gamma_2$  receptors exhibited high-affinity binding sites for [<sup>3</sup>H]muscimol and high affinity sites for the benzodiazepine [<sup>3</sup>H]Ro15-4513. However, the latter compound was only poorly displaced by the benzodiazepine agonist diazepam (10  $\mu$ M). This binding profile is very similar to that observed for  $\alpha_6\beta_2\gamma_2$  receptors and very different from  $\alpha_1\beta_2\gamma_2$  receptors, suggesting that the  $\alpha_4$  and  $\alpha_6$  subunits may share functional properties.

Regarding the sites of  $\alpha_4$  subunit gene expression, the  $\alpha_4$  mRNA is very abundant in most thalamic nuclei examined (for example, ventral posterior nucleus, medial geniculate nucleus) but is almost entirely absent from hypothalamus (Fig. 2). In thalamus, and also in hippocampus, the distribution of  $\alpha_1$  and  $\alpha_4$  mRNAs are well matched. However, their respective distributions in other brain regions suggest that the enclosed subunits occur in distinct receptor complexes. The  $\alpha_1$  and  $\alpha_4$  mRNAs have reciprocal distributions in the basal ganglia. The  $\alpha_4$  mRNA is more abundant than  $\alpha_1$  in the caudate nucleus. In contrast,  $\alpha_1$  mRNA predominates in globus pallidus. In addition,  $\alpha_1$  mRNA is very abundant in medial septum, an area where  $\alpha_4$  mRNA is absent. The  $\alpha_4$  transcript is absent or very rare in cerebellum and colliculi, both regions containing high amounts of  $\alpha_1$  mRNA.

These data suggest that the  $\alpha_4$  subunit participates in the formation of a previously uncharacterized native GABA<sub>A</sub> receptor which would fail to bind BZ agonists. Such a receptor subtype would be found mainly in forebrain/thalamic structures. Additionally, since the  $\alpha_4$  and  $\alpha_6$  polypeptides exhibit very similar functional properties, they can be grouped together in a subfamily of the  $\alpha$  subunits.

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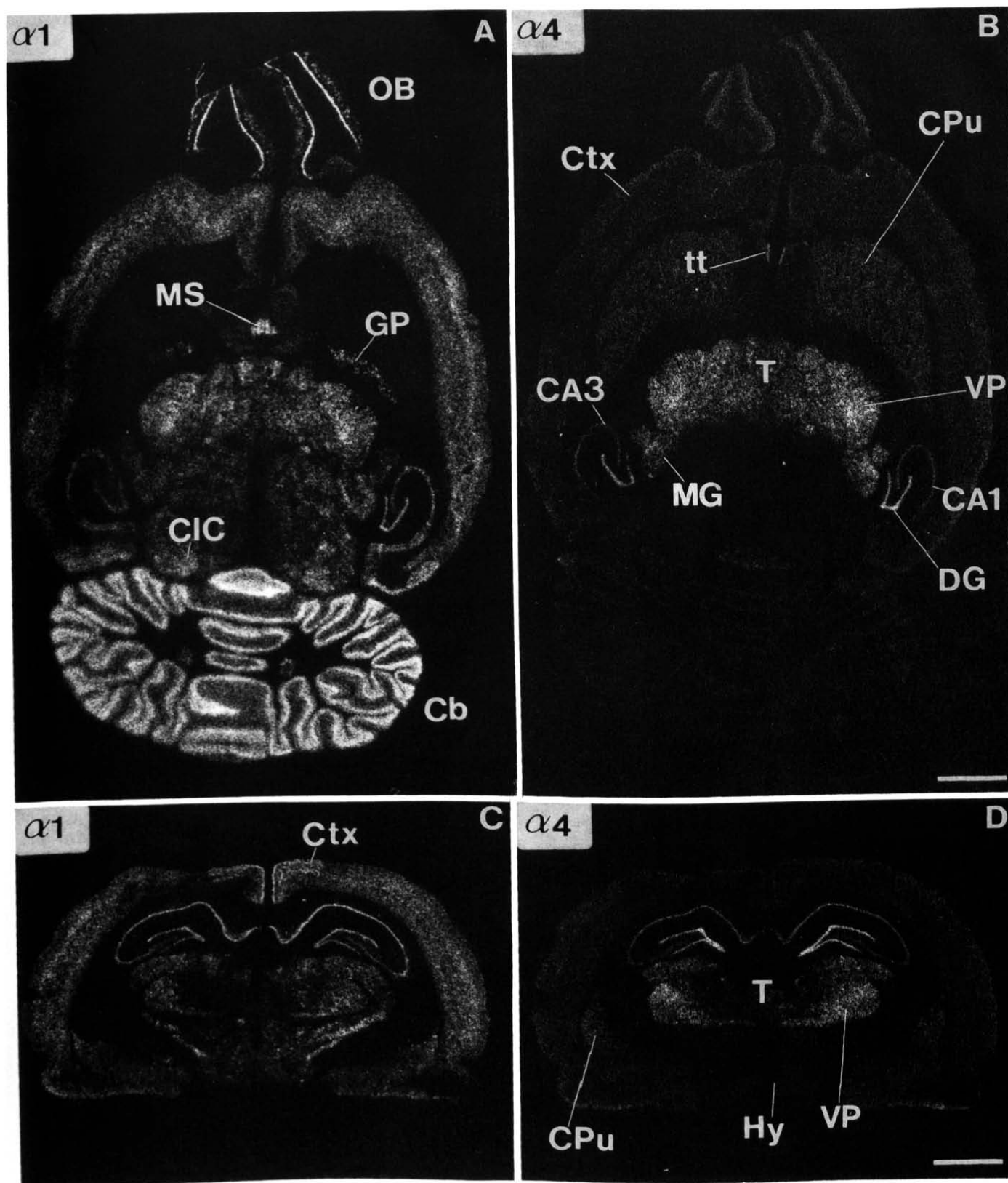


Fig. 2. Comparison of the distribution of  $\alpha_1$  and  $\alpha_4$  subunit mRNAs of the GABA<sub>A</sub> receptor by in situ hybridization. A.  $\alpha_1$ , horizontal section; B.  $\alpha_4$ , horizontal section; Cb, cerebellum; CIC, central nucleus of inferior colliculus; CPu, caudate putamen; C.  $\alpha_1$ , coronal section; D.  $\alpha_4$ , coronal section; Ctx, cortex; DG, dentate gyrus; GP, globus pallidus; Hy, hypothalamus; MG, medial geniculate; MS, medial septum; T, thalamus; tt, tenia tecta; VP, ventral posterior thalamic nucleus; Scale bar, (B, D) 2.4 mm.

Table I  
Comparison of binding properties of recombinant  $\alpha_4\beta_2\gamma_2$  GABA<sub>A</sub> receptors

	[ <sup>3</sup> H]Muscimol <i>K<sub>d</sub></i> (nM)	[ <sup>3</sup> H]Ro15-4513 <i>K<sub>d</sub></i> (nM)	Diazepam <i>K<sub>i</sub></i> (nM)	Flunitrazepam <i>K<sub>i</sub></i> (nM)	CI 218872 <i>K<sub>i</sub></i> (nM)	Flumazenil <i>K<sub>i</sub></i> (nM)
$\alpha_4\beta_2\gamma_2$	6.8 ± 1.9	4.97 ± 0.93	> 10000	> 10000	> 10000	107 ± 26
$\alpha_6\beta_2\gamma_2$	5 ± 0.5	5.4 ± 0.4	> 10000	> 10000	> 10000	90 ± 20
$\alpha_1\beta_2\gamma_2$	7 ± 2	15 ± 4	16 ± 1	2 ± 0.3	130 ± 40	0.5 ± 0.2

*K<sub>d</sub>* and *K<sub>i</sub>* values were calculated from IC<sub>50</sub> values [16]. Standard errors of means (SEM) derive from three independent experiments with values at different ligand concentrations determined in duplicate.

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