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Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α_4 subunit

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A cDNA of rat brain encoding the $GABA_A$ receptor α_4 subunit has been cloned. Recombinant receptors composed of α_4, β_2 and γ_2 subunits bind with high affinity the GABA agonist [^{3}H]muscimol and the benzodiazepine 'alcohol antagonist' [^{3}H]Ro 15-4513, but fail to bind benzodiazepine agonists. The α_4 subunit is expressed mainly in the thalamus, as assessed by in situ hybridization histochemistry, and may participate in a major population of thalamic $GABA_A$ receptors. The α_4 mRNA is found at lower levels in cortex and caudate putamen, and is rare in cerebellum.

$GABA_A$; Benzodiazepine receptor; In situ hybridization; Thalamus; α Subunit heterogeneity

1. INTRODUCTION

$GABA_A$ 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 $GABA_A$ receptor is assumed to be a pentameric structure composed of subunits belonging to subunit classes α, β, γ, δ and ρ [3,4]. So far, in the rodent fiva α subunit types have been identified by cDNA screening. These are α_1 [5], α_2 [6], α_3 [7], α_5 [5,7,8] and α_6 [9,10]. The subunit termed by us and others as α_5 [7,8] has also been termed as α_4 [5]. A cDNA encoding a bovine α_4 subunit has been isolated although not pharmacologically characterized [11], with the existence of a rat α_4 homologue remaining uncertain [11]. The α subunits are a major factor determining pharmacological diversity in $GABA_A$ receptors, with different α subunits combining with a β/γ pair to exhibit a broad spectrum of pharmacology [3,8,9,12]. Since it is important to analyze the whole repertoire of $GABA_A$ receptor subunits in an accessible experimental model, we have cloned the rat α_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.

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2. MATERIALS AND METHODS

2.1. *Isolation of cDNA clones*

A rat brain cDNA library constructed in λ-zap (Stratagene) was screened using a ^{32}P-labelled DNA fragment of the bovine α_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' ($r\alpha_{4\text{-}1}$), and 5'-TGTGTACCACATATCCCT-3' ($r\alpha_{4\text{-}2}$). 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 α_4 subunit.

2.2. *Pharmacology of recombinant $GABA_A$-benzodiazepine receptors*

Expression vectors for α_4, β_2 and γ_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'-CAAGTCGCCAGGCACAGGACGTGCAGGAGGGCGAGGCTGACCCCG-3') was constructed to a unique part of the α_4 subunit mRNA, complementary to amino acids 15 to 30 of the signal peptide. A 45 base α_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 [^{35}S]dATP (1200 Ci/mmol) to oligonucleotide. In situ hybridization was performed as described previously [15]. In brief, 14 μm 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 α_4 cDNA probe at high stringency resulted in the

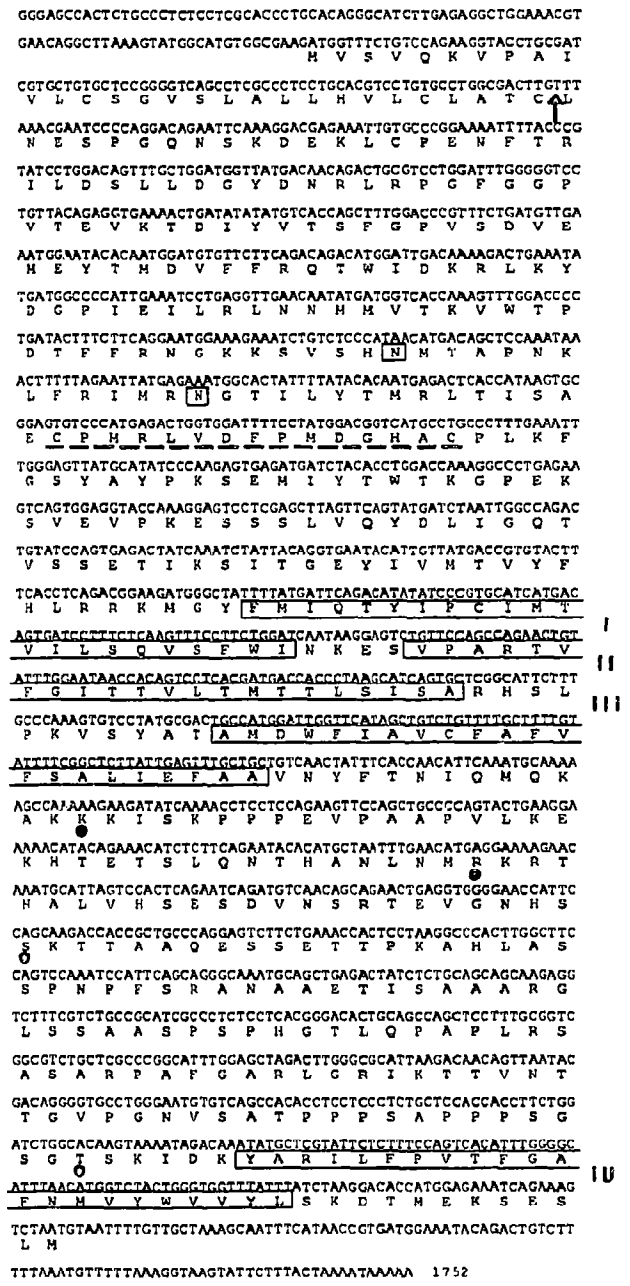

Fig. 1. Nucleotide and deduced amino acid sequence of the rat $GABA_A$ receptor α_4 subunit. The arrow marks potential cleavage site of signal peptide; N, potential *N*-glycosylation sites; filled circles, potential protein kinase C phosphorylation sites; open circles, potential cAMP dependent protein 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 α_4 subunit [5]. Like its bovine counterpart, the rat α_4 polypeptide is predicted to contain a large cytoplasmic portion, making α_4 the largest $GABA_A$ 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 α_4 subunit was examined by co-expression with β_2 and γ_2 subunits in cultured mammalian 293 cells (Table I). The $\alpha_4\beta_2\gamma_2$ receptors exhibited high-affinity binding sites for [^{3}H]muscimol and high affinity sites for the benzodiazepine [^{3}H]Ro15-4513. However, the latter compound was only poorly displaced by the benzodiazepine agonist diazepam (10 μM). 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 α_4 and α_6 subunits may share functional properties.

Regarding the sites of α_4 subunit gene expression, the α_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 α_1 and α_4 mRNAs are well matched. However, their respective distributions in other brain regions suggest that the enclosed subunits occur in distinct receptor complexes. The α_1 and α_4 mRNAs have reciprocal distributions in the basal ganglia. The α_4 mRNA is more abundant than α_1 in the caudate nucleus. In contrast, α_1 mRNA predominates in globus pallidus. In addition, α_1 mRNA is very abundant in medial septum, an area where α_4 mRNA is absent. The α_4 transcript is absent or very rare in cerebellum and colliculi, both regions containing high amounts of α_1 mRNA.

These data suggest that the α_4 subunit participates in the formation of a previously uncharacterized native $GABA_A$ receptor which would fail to bind BZ agonists. Such a receptor subtype would be found mainly in forebrain/thalamic structures. Additionally, since the α_4 and α_6 polypeptides exhibit very similar functional properties, they can be grouped together in a subfamily of the α subunits.

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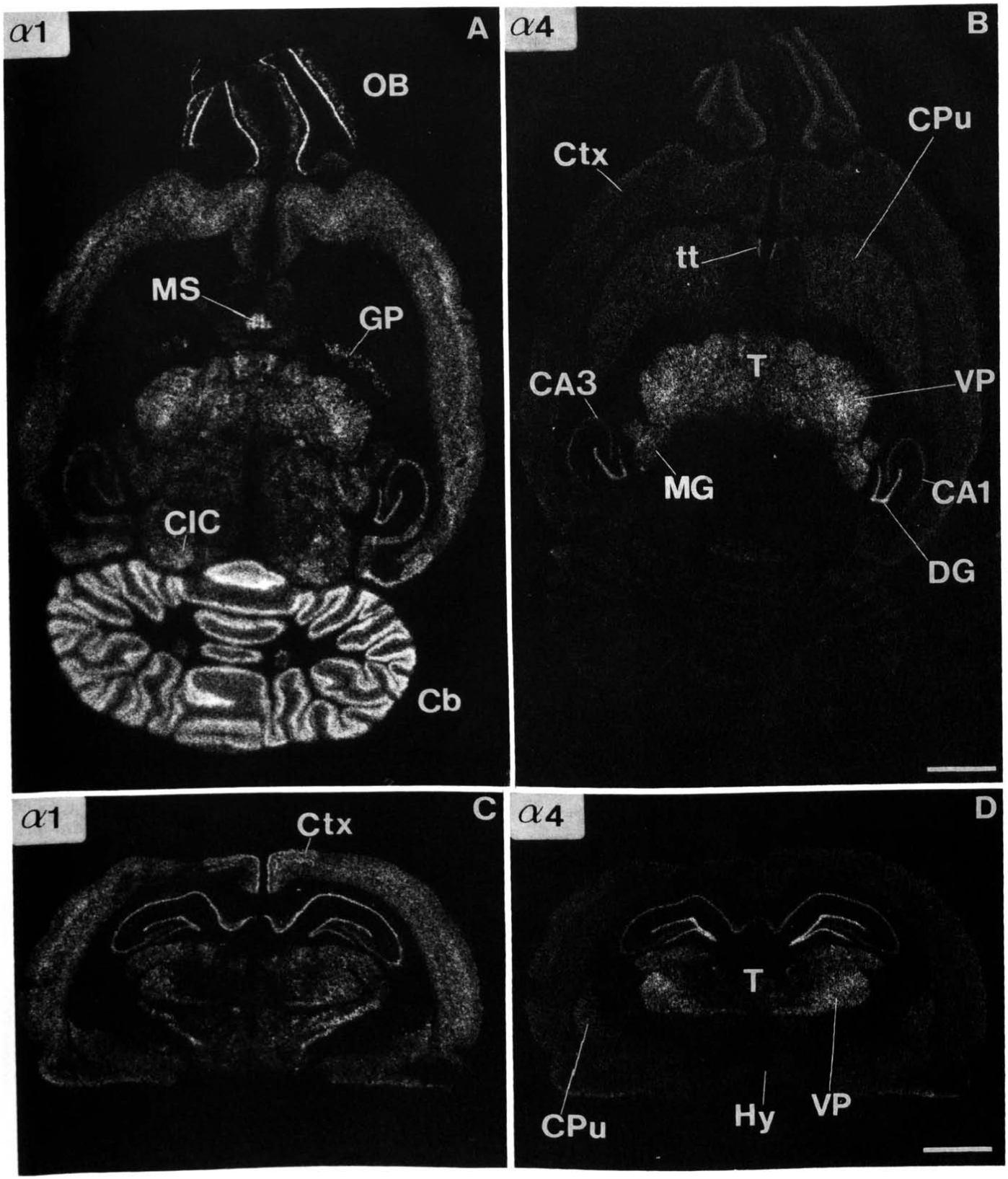

Fig. 2. Comparison of the distribution of α_1 and α_4 subunit mRNAs of the $GABA_A$ receptor by in situ hybridization. A. α_1, horizontal section; B. α_4, horizontal section; Cb, cerebellum; CIC, central nucleus of inferior colliculus; CPu, caudate putamen; C. α_1, coronal section; D. α_4, coronal section; 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 $\alpha_x\beta_2\gamma_2$ $GABA_A$ receptors

	[^{3}H]Muscimol K_d (nM)	[^{3}H]Ro15-4513 K_d (nM)	Diazepam K_i (nM)	Flunitrazepam K_i (nM)	Cl 218872 K_i (nM)	Flumazenil K_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_d and K_i values were calculated from IC_{50} 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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