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Functional expression and sites of gene transcription of a novel α subunit of the $GABA_A$ receptor in rat brain

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Two α subunits of the $GABA_A$ receptor in rat brain have been identified by molecular cloning. The deduced polypeptide sequences share major characteristics with other chemically gated ion channel proteins. One polypeptide represents the rat homologue of the α3 subunit previously cloned from bovine brain [14], while the other polypeptide is a yet unknown subunit, termed α5. When coexpressed with the β1 subunit in *Xenopus* oocytes the receptors containing the α5 subunit revealed a higher sensitivity to GABA than receptors expressed from α1 + β1 subunits or α3 + β1 subunits (K_a = 1 μM, 13 μM and 14 μM, respectively). The α5 subunit was expressed only in a few brain areas such as cerebral cortex, hippocampal formation and olfactory bulb granular layer as shown by in situ hybridization histochemistry. Since the mRNA of the α5 subunit was colocalized with the α1 and α3 subunits only in cerebral cortex and in the hippocampal formation the α5 subunit may be part of distinct $GABA_A$ receptors in neuronal populations within the olfactory bulb.

$GABA_A$ receptor heterogeneity; $GABA_A$ receptor isoform α5/β1; $GABA_A$ receptor isoform α3/β1; Hybridization histochemistry, in situ; Subunit expression; (*Xenopus* oocytes)

1. INTRODUCTION

$GABA_A$ receptors, the major synaptic targets of the neurotransmitter GABA in the CNS, constitute gated chloride channels. By virtue of their allosteric modulation by drugs such as barbiturates and benzodiazepines, they serve as pharmacological control elements for the regulation of anxiety, vigilance, muscle tension and epileptiform activity [1,2]. Based on photoaffinity labeling [3,4] and receptor purification [5–7] two polypeptides, the α and β subunit, were originally identified as major constituents. Molecular cloning of subunit cDNAs, however, revealed an unexpected subunit heterogeneity comprising at least four classes of subunits (α,β,γ,δ) which share an amino acid sequence identity between 35 and 40% [8–13]. Each class of subunits may comprise several variants which share a close sequence homology (70–75%). So far, three α subunits (α1, α2, α3) [14], three β subunits (β1, β2, β3) [9], one γ subunit (γ2) [11,12] and one δ subunit [10] have been identified. We now report on the cloning, functional expression and mRNA distribution of a further α subunit variant in rat brain.

2. MATERIALS AND METHODS

2.1. *Isolation of cDNA clones*

A λgt11 rat brain cDNA library [13] was screened with a 2700-bp ^{32}P-nick-translated fragment encoding the entire bovine $GABA_A$ receptor α1 subunit [8]. The positive cDNA clones were subcloned into the M13RF vector mp19 (Pharmacia) before sequencing. An overlapping set of deletions was generated using the exonuclease III method described by Henikoff [15]. DNA sequencing was performed by the chain-termination technique of Sanger et al. [16]. Sequence analysis was performed on a Vax terminal using the GCG program (Genetics Computer Group, University of Wisconsin).

2.2. *Expression of cloned subunits in Xenopus oocytes*

The rat α1 (1503 bp), α3 (3800 bp), α5 (2000 bp) and β1 (1941 bp) cDNAS were cloned into the pSpT19 vector (Pharmacia) for transcription. The cRNAs were capped and polyadenylated as previously described [13]. Follicle cells from *Xenopus laevis* were mechanically isolated, maintained in culture, and, on the following day, microinjected with about 50 nl of a solution containing each cRNA at a concentration of 200 nM. The follicular cell layers were removed as described [17] 1 day before the electrophysiological measurements. Oocytes were placed on a nylon grid in a 0.4 ml bath, which was perfused throughout the experiment at 6 ml/min with 90 mM NaCl/1 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/5 mM Hepes-NaOH (pH 7.4). The effect of diazepam was tested using a fast perfusion technique, which permitted medium exchanges in the subsecond time range [12]. All experiments were carried out at room temperature (22–26°C). For the current measurements the oocytes were impaled with two microelectrodes and the membrane potential voltage-clamped at −100 mV as described [17]. Current amplitudes were read as the peak currents. Dose–response curves were fitted using a nonlinear least-squares method (Gauss–Newton–Marquardt).

2.3. *In situ hybridization histochemistry*

2.3.1. Tissue preparation

Male rats (specific pathogen-free (SPF) albino, Füllinsdorf, Switzerland) weighing 120–130 g were decapitated, the brains removed, then immediately frozen on dry ice and stored at −80°C until used. Cryostat sections (12 μm) were mounted on slides, previously coated twice with 0.5% gelatin + 0.5% $CrK(SO_4)_2$, then fixed in 4% paraformaldehyde (in PBS pH 7.4) for 40 min followed by three 5 min washes in PBS, then stored at −20°C until used.

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2.3.2. Oligonucleotide labelling

Subunit-specific synthetic oligodeoxyribonucleotide probes (Med prob A.S.) representing sense and antisense sequences of the rat $\alpha 1$ subunit (bp 1144–1197) [13], the $\alpha 3$ subunit (bp 1535–1585) and the $\alpha 5$ subunit (bp 1486–1537) were labelled at the 3′ end using terminal deoxynucleotidyl transferase (BRL) and ^{35}S-dATP (New England Nuclear). The reaction mixture (30 μl total) contained 20 μl ^{35}S-dATP, 6 μl tailing buffer (BRL: 500 mM potassium cacodylate pH 7.2, 10 mM $CoCl_2$, 1.0 mM DTT) and 1 μl terminal deoxynucleotidyl transferase (15 U/μl). The cocktail was transferred to 37°C for 5 min, then the reaction was stopped by adding 70 μl spun-column buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5) to a final volume of 100 μl. The labelled probe was separated from unincorporated nucleotides with a Sephadex G50 spun-column (4 min at 1600 × g, Sorvall SW 24).

2.3.3. In situ hybridization

Brain sections were brought to room temperature for 1 h before

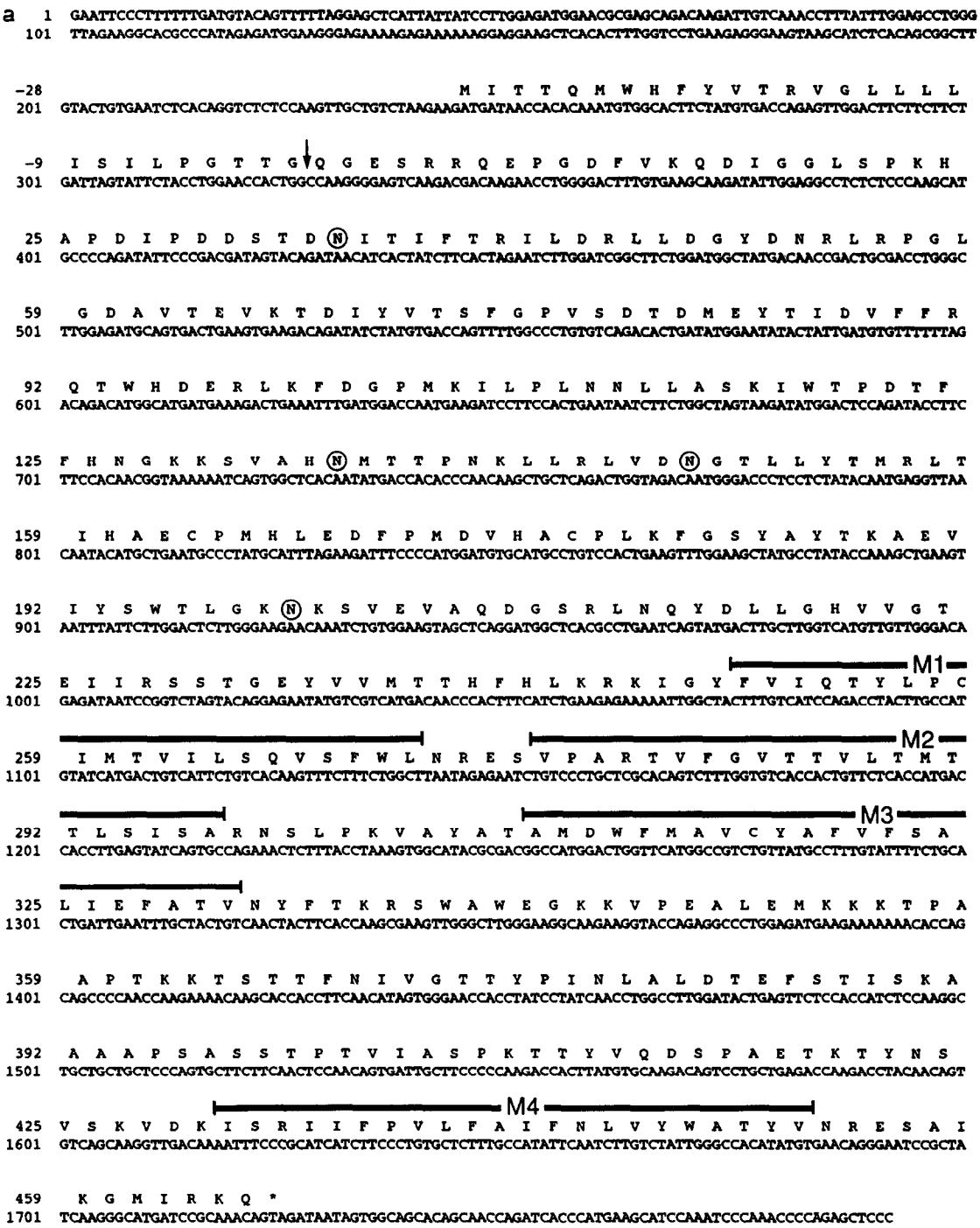
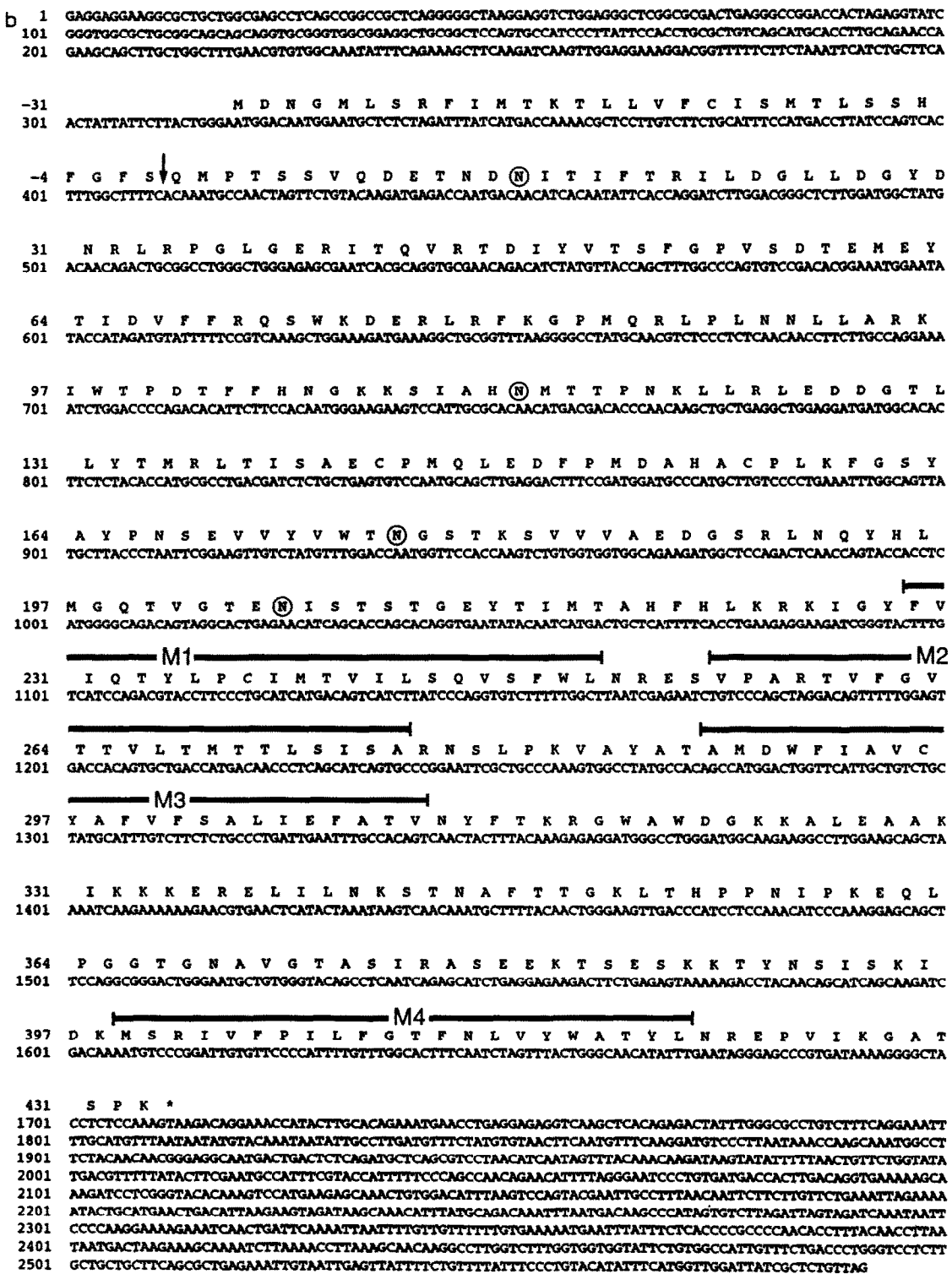


Fig.1. Nucleotide and deduced amino acid sequence of the $\alpha 3$ subunit (a) and the $\alpha 5$ subunit (b) of the $GABA_A$ receptor in rat brain. Potential sites of N-linked glycosylation are encircled. The proposed membrane spanning domains (M1–M4) are indicated by solid lines. The arrow points to the putative signal sequence cleavage site.

carrying out a prehybridization step consisting of a 1 h incubation at room temperature in 50 μl of a solution containing 4 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0), 2 mg/ml BSA, 10% dextransulfate, 1 mg/ml *t*-RNA (Boehringer Mannheim), 1 mg/ml Poly A (Sigma), 50% deionized formamide (Bethesda Research Laboratories) and 10 mM dithiothreitol (Fluka) under Parafilm coverslips. For the hybridization step, labelled probe (1.5×10^6 cpm in 50 μl per section) was added and incubated in a moist chamber at 30°C for 20–24 h. After rinsing the sections briefly in 2 × SSC/50% deionized formamide and 10 mM dithiothreitol (first for 15 min at room temperature, then three times for 15 min at 40°C) and afterwards in 2 × SSC and 10 mM dithiothreitol (three times for 1 h at room temperature), the sections were dehydrated in ethanol, exposed to sheet film (Hyperfilm, β/Max, Amersham) and then dipped



in a nuclear track emulsion (Ilford (Warnington, PA) K5, diluted 1:1 with distilled water) to reveal the regional and cellular localization of the α subunit subtypes mRNAs.

3. RESULTS AND DISCUSSION

Screening of a rat brain cDNA library with the bovine α1 subunit cDNA probe containing the entire coding sequence [8] resulted in the isolation of six positive clones, of which two λoTα2F 3800 bp and λoTRαF3 3000 bp hybridized only weakly but displayed distinct patterns in restriction mapping. The clone λoTα2F (1792 bp) contained an open reading frame of 1468 nucleotides encoding a protein of 493 amino acid residues (fig.1a). Its deduced amino sequence revealed a 96% sequence identity with bovine α3 subunit suggesting that this clone is the rat homologue of the α3 subunit. The sequence homology with the rat α1 and bovine α2 subunits amounted to 71% and 72%, respectively.

The clone λoTαF3 (2591 bp; fig.1b) encoded a polypeptide of 433 amino acid residues (48 kDa). It displayed a striking sequence homology to the known α subunit variants. Its sequence identity with the rat α1 subunit [8] was 70%, with the bovine α2 subunit [14] 74% and with the rat α3 subunit 69%. These findings suggest that the isolated clone codes for a novel α subunit variant, which was termed α5.

The structural features of the deduced polypeptides of the α3 and α5 subunits conformed with those of other subunits of the $GABA_A$-receptor [19]. Potential N-linked glycosylation sites (four each in α3 and in α5) and potential disulfide-bonded loops are located on the presumed extracellular domain. Hydropathy analysis revealed four putative membrane-spanning regions (M1–M4). The domains of M1–M3 are virtually identical between the rat α1, α3 and α5 subunits with only one conservative amino acid change (I→M314) in the α3 sequence. The lowest degree of sequence homology was found in the signal peptides [20], in the most extracellular N-terminus and in the presumed cytoplasmic loops connecting the M3 and M4 domains.

In order to test whether the new α5 subunit variant differed functionally from the known members of the α subunit family, the α5 subunit RNA was coexpressed with the rat β1 subunit RNA in *Xenopus* oocytes. The resulting receptors were compared to those coexpressed from either $\alpha1+\beta1$ or $\alpha3+\beta1$ subunit RNAs. Upon superfusion with GABA, inward currents were recorded under voltage clamp conditions. Dose- response studies revealed (fig.2) that the receptor expressed from $\alpha5+\beta1$ RNAs had a higher sensitivity to GABA than the receptors expressed from either $\alpha1+\beta1$ RNAs or $\alpha3+\beta1$ RNAs. The dose–response curves were fitted with the equation $I(c)=I_{max}c^n/(c^n+K_a^n)$, where I_{max} is the maximal current amplitude, c is the GABA concentration, n the Hill coefficient, and K_a the GABA concentration that elicits half-maximal current response. The best fit indicated K_a values of 13 μM, 14 μM and 0.97 μM for the combinations of the β1 subunit with the

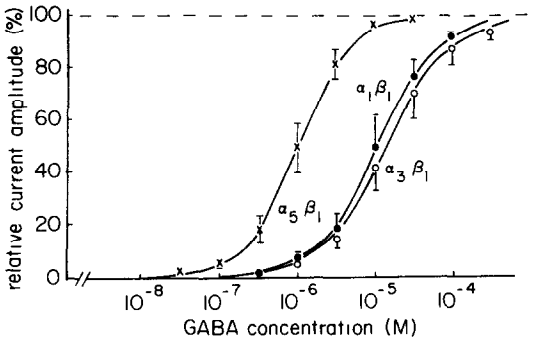


Fig.2. GABA dose–response curves recorded from *Xenopus* oocytes injected with RNAs coding for either $\alpha1+\beta1$ subunits (●—●), $\alpha3+\beta1$ subunits (○—○) or $\alpha5+\beta1$ subunits (×—×). For each subunit combination the dose–response curve was obtained at least three times in oocytes exhibiting different degrees of channel expression. Each curve was fitted with the equation given in the text. Maximal current was arbitrarily set at 100% and the current amplitudes were expressed in relative values. The curves shown were obtained by reapplying the fit to the standardized averaged values.

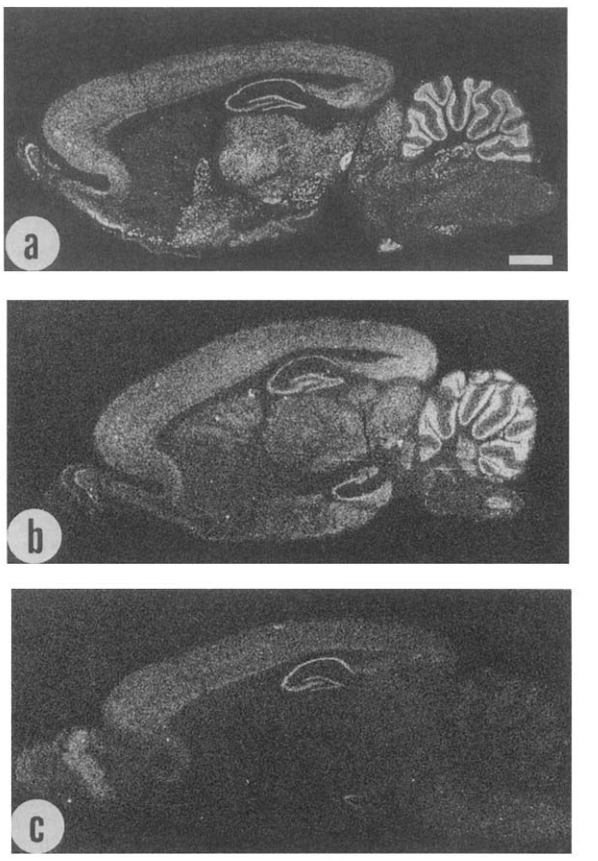


Fig.3. In situ hybridization with ^{35}S-labeled antisense probes of the α1 subunit (a), α3 subunit (b) and α5 subunit (c) in parasagittal sections of rat brain. White areas represent regions fo hybridization Bar = 2 mm.

$\alpha 1$, $\alpha 3$ and $\alpha 5$ subunits, respectively. Thus, the $\alpha 5$ subunit appears to confer a differential sensitivity to GABA-gating of the channel. However, for all subunit combinations studied some variability in the individual K_a values determined in different oocytes was observed even when oocytes from the same batch of cells were used. The variability was most prominent for the combination of $\alpha 3+\beta 1$ subunits. In this case a K_a value of 14 μM was deduced from three individual dose–response curves as averaged in fig.2. Two additional dose– response curves indicated a K_a in the submicromolar range. The reason for this diversity is not clear. Possibly different oocytes assemble ion channels with different subunit stoichiometries. When GABA-gated channels are expressed from a triple-combination of subunits, $\alpha 1+\beta 1+\gamma 2$, no such variability in the dose–response curves is observed [12].

The limiting slopes in log/log plots of the dose–response curves shown in fig.2 revealed Hill coefficients of 0.9, 0.9 and 1.2 for the combinations of the $\beta 1$ subunit with the $\alpha 1$, $\alpha 3$ and $\alpha 5$ subunits, respectively. The current response elicited by GABA was not potentiated by 1 μM diazepam in any of the subunit combinations tested.

Injection of $\beta 1$ subunit RNA leads to the formation of anion channels which are open in the absence of GABA [21]. Co-injection with either $\alpha 1$, $\alpha 3$ or $\alpha 5$ subunit RNA prevented the formation of these channels. Thus, the assembly of heteroligomeric channels appears to be favoured over the formation of homomeric channels.

Among the known α subunit variants, the $\alpha 1$ subtype has a very wide distribution in rat brain. This was shown earlier by in situ hybridization histochemistry using a cRNA probe [18] and was now confirmed using a subunit-specific oligonucleotide probe (fig.3a). The brain regions containing $\alpha 1$-mRNA include the olfactory bulb mitral cells, cerebral cortex (layers II, III, V, VI), anterior olfactory nucleus, hippocampal pyramidal cells, dentate gyrus granule cells, various thalamic nuclei, colliculi, cerebellar Purkinje cells, stellate, basket and granule cells, deep cerebellar nuclei (n. interpositus), brainstem nuclei (e.g. vestibular, facial, motor trigeminal). In order to explore the physiological relevance of the $\alpha 3$ and $\alpha 5$ subunit variants in rat brain, their pattern of gene expression was visualized in brain sections using sequence specific ^{35}S-labeled sense and antisense oligonucleotide probes. While the sense probes resulted only in background labeling, the antisense probes revealed a striking hybridization pattern. The mRNA for the $\alpha 3$ subunit (fig.3b) was codistributed with $\alpha 1$ in most brain regions, although the intensity of the hybridization signal was considerably lower than that of $\alpha 1$. Notable exceptions which did not reveal an $\alpha 3$-signal include the cerebellar Purkinje cells and cells in the molecular layer, the pallidum and substantia nigra pars reticulata. A weak $\alpha 3$ hybridization signal but no $\alpha 1$ signal was observed in caudate-putamen and substantia nigra zona compacta. The $\alpha 5$ mRNA (fig.3c) was more restricted in its distribution than $\alpha 3$ mRNA. Its hybridization signal codistributed with the $\alpha 1$ and $\alpha 3$ signal only in cerebral cortex layers (particularly layer VI) and in the hippocampal formation. A weak hybridization signal was additionally observed in the olfactory bulb granular layer in which neither $\alpha 1$ nor $\alpha 3$ signals were detected. Our results show that the genes of the $\alpha 3$ and $\alpha 5$ subunits can be differentially regulated and may contribute to distinct $GABA_A$-receptors in different neuronal populations. These receptors may display a differential sensitivity to GABA.

REFERENCES

[1] Haefely, W., Kyburz, E., Gerecke, M. and Mohler, H. (1985) Adv. Drug Res. 14, 165–322.
[2] Haefely, W. (1989) in: Therapeutic Implications (E.A. Barnard, E. Costa eds) Raven, New York, pp. 47–70.
[3] Mohler, H., Battersby, M.K. and Richards, J.G. (1980) Proc. Natl. Acad. Sci. USA 77, 1666–1670.
[4] Sieghart, W. and Karobath, M. (1980) Nature 286, 285–287.
[5] Schoch, P., Häring, P., Takacs, B., Stähli, C. and Mohler, H. (1984) J. Recept. Res. 4, 189–200.
[6] Sigel, E., Stephenson, F.A., Mamalaki, C. and Barnard, E.A. (1983) J. Biol. Chem. 285, 6965–6971.
[7] Sigel, E. and Barnard, E.A. (1984) J. Biol. Chem. 259, 7219–7223.
[8] Schofield, P.R., Darlison, M.G., Fujita, N., Burt, D.R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, V.R., Glencorse, T., Seeburg, P.H. and Barnard, E.A. (1987) Nature 328, 221–227.
[9] Ymer, S., Schofield, P., Draguhn, A., Werner, P., Köhler, M. and Seeburg, P.H. (1989) EMBO J. 8, 1665–1670.
[10] Shivers, B.D., Killisch, I., Sprengel, R., Sontheimer, H., Köhler, M., Schofield, P.R. and Seeburg, P. (1989) Neuron 3, 327–337.
[11] Pritchett, D.B., Sontheimer, H., Shivers, B.D., Ymer, S., Kettenmann, H., Schofield, P.R. and Seeburg, P.H. (1989) Nature 338, 582–585.
[12] Malherbe, P., Sigel, E., Baur, R., Persohn, E., Richards, J.G. and Möhler, H. (submitted).
[13] Malherbe, P., Draguhn, A., Multhaup, G., Beyreuther, K. and Möhler, H. (submitted).
[14] Levitan, E.S., Schofield, P.R., Burt, D.R., Rhee, L.M., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H.F., Stephenson, S., Darlison, M.G., Barnard, E.A. and Seeburg, P.H. (1988) Nature 335, 76–79.
[15] Henikoff, S. (1984) Gene 28, 351–359.
[16] Sanger, F., Coulson, A., Barnell, B., Smith, A. and Roe, B. (1980) J. Mol. Biol. 143, 161–178.
[17] Sigel, E. (1987) J. Physiol. (Lond.) 386, 73–90.
[18] Sequier, J.M., Richards, J.G., Malherbe, P., Price, G.W., Matthews, S. and Mohler, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7815–7819.
[19] Barnard, E.A., Darlison, M.G. and Seeburg, P. (1987) Trends Neurosci. 10, 502–509.
[20] Von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690.
[21] Sigel, E., Baur, R., Malherbe, P. and Möhler, H., FEBS Lett. (in press).