GABA_A receptor β subunit heterogeneity: functional expression of cloned cDNAs

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Cloned cDNAs encoding two new β subunits of the rat and bovine GABA_A receptor have been isolated using a degenerate oligonucleotide probe based on a highly conserved peptide sequence in the second transmembrane domain of GABA_A receptor subunits. The $\beta 2$ and $\beta 3$ subunits share $\sim 72\%$ sequence identity with the previously characterized $\beta 1$ polypeptide. Northern analysis showed that both $\beta 2$ and $\beta 3$ mRNAs are more abundant in the brain than β 1 mRNA. All three β subunit encoding cDNAs were also identified in a library constructed from adrenal medulla RNA. Each β subunit, when co-expressed in *Xenopus* oocytes with an α subunit, forms functional GABAA receptors. These results, together with the known α subunit heterogeneity, suggest that a variety of related but functionally distinct GABA_A receptor subtypes are generated by different subunit combinations.

Key words: GABA_A receptor/ β subunit/receptor subtypes/ molecular cloning/oocyte expression

Introduction

GABA (γ -aminobutyric acid), the major inhibitory neurotransmitter in the vertebrate brain, mediates neuronal inhibition by opening a chloride channel integral to the GABA_A receptor, which is also the target for a variety of therapeutically important drugs (reviewed in Olsen and Venter, 1986). Affinity-purified receptor is electrophoretically resolved into two major bands (α , 48-53 kd and β , 55-57 kd). The smaller band can be photoaffinitylabelled by benzodiazepine derivatives and the larger one by GABA agonists (reviewed by Stephenson, 1988). Molecular cloning of cDNAs encoding GABA_A receptor subunits was facilitated by peptide sequences derived from purified receptor (Schofield et al., 1987; Levitan et al., 1988). Analysis of these cDNAs established that the ' α band' is heterogeneous, consisting of several variants of the α subunit (Levitan et al., 1988) and of other subunits (Pritchett et al., 1989). This confirmed the molecular heterogeneity of GABAA receptors, postulated on the basis of pharmacological (Squires et al., 1979; Braestrup and Nielsen, 1981; Unnerstall et al., 1981; Cooper et al., 1987) and photoaffinity labelling (Möhler *et al.*, 1980; Sieghart *et al.*, 1983; Fuchs *et al.*, 1988) studies. However, the ' β band' was regarded as being homogeneous (Häring *et al.*, 1985; Mamalaki *et al.*, 1987). This band is so far molecularly characterized by only one cloned cDNA that encodes a subunit (β 1) with significant sequence similarity to the α subunits and, when co-expressed with these in *Xenopus* oocytes, produces functional GABA_A receptors (Schofield *et al.*, 1987; Levitan *et al.*, 1988). Sequence homology also extends, in part, to other ligand-gated ion channels, reflecting the existence of a receptor superfamily (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987a).

Comparison of the polypeptide sequences of the three GABA_A receptor α subunits, the β 1 subunit as well as the 48 kd subunit of the glycine receptor revealed that the highest sequence identity resides in the four putative transmembrane segments (M1-M4). In particular, a contiguous sequence of eight amino acids rich in threonines is found in M2 of all these polypeptides (Grenningloh et al., 1987b) which, by analogy to M2 of nicotinic acetylcholine receptor (Imoto et al., 1988; Leonard et al., 1988), is thought to form part of the channel lumen. We have used a highly degenerate oligonucleotide probe encoding this peptide sequence to screen for additional GABA_A receptor subunits. We report the isolation of two new β subunit encoding cDNAs, β 2 and β 3, from both rat and bovine brain cDNA libraries and show that these new β subunits are more abundant in brain than the β 1 subunit. Functional expression in *Xenopus* oocytes demonstrates that these β subunits are capable of combining with an α subunit to form GABA_A receptor chloride channels.

Results

Cloning of β 2 and β 3 subunit cDNAs

A cDNA library constructed from bovine brain RNA was screened with the degenerate oligonucleotide probe and numerous hybridizing signals were obtained. Among these were cDNAs encoding the known GABA_A receptor subunits $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ (Schofield *et al.*, 1987; Levitan *et al.*, 1988), which were identified by subunit specific oligonucleotides. The remaining hybridizing clones were sequenced. Preliminary sequence data were obtained either directly from λ DNA or from recombinant M13 DNA, using the degenerate oligonucleotide as a primer. Clones encoding new subunits were identified by homology of their deduced amino acid sequence with the third transmembrane region of previously characterized GABA_A receptor subunits.

Two cDNA clones were isolated that encoded different polypeptides showing high sequence identity to the GABA_A receptor β 1 subunit. These polypeptides were designated as β 2 and β 3 subunits. By comparison with the β 1 polypeptide, the encoded β 2 subunit sequence lacked an initiation codon and the β 3 subunit sequence lacked the N-terminal 45 amino acids of the complete polypeptide. Full-length cDNA clones were not identified upon rescreening, but a complete β 3 subunit encoding clone was isolated from a bovine adrenal gland cDNA library (see below). We then screened a rat forebrain cDNA library with oligonucleotides based on the bovine β subunit coding sequences and isolated full-length cDNA clones for the rat β 1, β 2 and β 3 subunits. The nucleotide and deduced amino acid sequences of the rat subunits, including part of the 5' and 3' non-coding regions, are shown in Figure 1. All three cDNAs encode polypeptides of ~450 amino acid residues (M_r 52 kd) with a 25-residue signal peptide (Von Heijne, 1986). The predicted mature polypeptide sequences contain four putative membrane-spanning regions, three potential N-linked glycosylation sites and a 15-residue-long, cysteine-flanked region, characteristic

а	- 25	TCCCGAATGACCTGCCGCCGACTAAGTTGCATTCCTTGAATCTTCGCAGAAAGACAATTCTTTCATCAGAGTTAGTAATGTGGACAGTAC M W T V Q	90 - 21
	91	AAAATCAAGAGAGTITGGGGCTTCTCTCTCTCTGTGAGGTGGCTGCCAGGGTTGTTGTGGCACAGCACCAGCAACAACATGT	180
	- 20	N R E S L G L L S F P V M V A M V C C A TH S S N E P S M M S	10
	181	CATACGTGAAAGAGAGTGGACCGGACTGCTCAAAGGATATGACATTGCGTTGCGGCCAGACTTTGGAGGGCCCCCGGTGGACGTCGGGA	270
	11	Y V K E T V D R L L K G Y D 1 R L R P D F G G P P V D V G M	40
	271	TGCGGATCGATGTCGCCAGCATAGACATGGTCTCGGAAGTGAATATGGATTATACACTCACCATGTATTTCCAGCAGTCTTGGAAGGACA	360
	41	R I D V A S I D M V S E V N M D Y T L T M Y F Q Q S W K D K	70
	361	AGAGACTTICATATTCTGGAATCCCATTAAATCTGACCCTTGACAACAGGGTAGCTGACCAACTCTGGGTACCAGACACCAGCTACTTTCTCA	450
	71	R L S Y S G I P L N L T L D N R V A D Q L N V P D T Y F L N	100
	451	ATGACAAGAAGTEATTIGTGCATGGGGTCCAGGGAAAAAACCGAATGATCAGGCTTCATCCTGATGGAACTGTTCTCTATGGACTACGGA	540
	101	D K K S F V H G V T V K N R H I R L H P D G T V L Y G L R I	130
	541	TCACAACCACCTGCAAGCTGCATGATGGATCTCCGCAAGATACCCCTCGGATGAGCAAAACTGCACCCTGGAGATCGAAAGTTATGGCTATA	630
	131	T T T A A C N N D L R R Y P L D E O N C T L E I E S Y G Y T	160
	631	CCACCGATGACATCGAATITTACTGGAATGGAGGAGGAGGAGGAGCAGCAACTGGGGTCAATAAAATTGAGCTTCCCCCAATITTCAATTGTCG	720
	161	T D D I E F Y W N G G E G A V T G V N K I E L P O F S I V D	190
	721 191	ACTACAAGATGGTGTCCAAGAAGGTGGAATTCACAACAGGGGCATATCCACGACTATCACTAAGTTTTCGTCTAAAGAGAAACATCGGTT $Y \ K \ M \ V \ S \ K \ K \ V \ E \ F \ T \ G \ A \ Y \ P \ R \ L \ S \ L \ S \ F \ R \ L \ K \ R \ N \ I \ G \ \underline{Y}$	810 220
	811	ACTTCATTITGCAGACCTACATGCCCTCCACACTGATTACAATTCTGTCCTGGGTGGTCGTTTTGGATCAACTATGATGCATCTGCAGCCA	900
	221	FILOTYNPSTLITILSNYSFNINYDASAAR	250
	901	GAGTEGECETAGGAATEACEAEGGTGETGAEEATGAEAACEATEAGTAETEAGTAECETEAGGGAGAETETGEEAAAGATECETTAEGTEAAAG	990
	251	<u>V A L G I T T V L T M T T I S T H L</u> R E T L P K I P Y V K A	280
	991	CGATTGATATCTATCTGATGGTTGTTTTGTGTTCGTGTTCCTGGCCTTACTGGAGTACGCCTTTGTAAATTACATTTTCTTCGGAAAAG	1080
	281	<u>1 D I Y L M G C F V F V F L A L L E Y A F V</u> N Y I F F G K G	310
	1081	GCCCTCAGAAAAAAGGAGGGAGGAAACAAGACCAGAGTGCCAATGAAAAGAACAAACTGGAGATGAACAAAGTCCAAGGTCAAGGTCCACG	1170
	311	P Q K K G A S K Q D Q S A N E K N K L E N N K V Q V D A H G	340
	1171	GCANTATICICCICAGCACCCIGGAAATCAGGAATGAGACCAGIGGCICCGAGGGGCICCAACGGGGGGAAGGGACCCCAAGGCCACTAIGI	1260
	341	NILLSILEIRNEISSEVLIGVSDPKATMY	370
	1261	ACTCATACGACAGCGCCAGCATCCAGTACCGCGAAGCAGCGGTGGGGGCTTCGGACGAGGGGCTGGACAGGGGGTGCCG	1350
	371	S Y D S A S I O Y R K P L S S R E G F G R G L D R H G V P G	400
	1351	GCAAGGGTCGCATCCGCAGACGTGCCCTCAGCTCAAGTCGCTGAACTCGACGTGAACTCCCTAGACAAGTGGTCCCGAA	1440
	401	K G R I R R R A S G L K V K I P D L T D V N S I D K <u>W S R M</u>	430
	1441	TGTTCTTCCCCATCACATTTTCCCTTTATAGTGGGTTTAGTATTGGCTTTACTATGTAGACTCAAGTTCAGTCTCATGGTTCGGTTTAGACTC	1530
	431	<u>F F P I T F S L F N V V V U L Y V</u> H	449
	1531 1621 1711 1801 1891	CTTTCCTCCTCGCTTGTTTTTAACCCCACAGATTTCCAACAGTGGTACTGCTATGGTTTTGAGGTAAGAGTTCGGCCTTCCAGTG GTTTCGATCTGTGTGTGATTTTTATCACACACATGTTACTTCACACACA	1620 1710 1800 1890

of all subunits of ligand-gated ion channels (Criado *et al.*, 1986; Schofield *et al.*, 1987). Notably, all three β subunits contain a cAMP-dependent phosphorylation site in a homologous position within their putative intracellular domain, suggesting involvement in the cellular control of receptor activity.

Comparison of the three β polypeptides predicted from either rat or bovine cDNAs (Figure 2) shows that 72% of the residues are invariant, reflecting an identity similar to that seen between different α subunits (Levitan *et al.*, 1988). As for the α variants, regions of highest homology include the membrane-spanning domains and large extracellular region. Low sequence similarity is seen in the signal peptides and in the intracellular domain located between M3 and M4.

b	1	GAATTACTGCACTGGGCAGACTAAGTTGGATCTCCTCTTCTCAGTGAATCCCTCAATCCCACCAAAAACTAAAGGGATCTGGAGAGTCCG	90
	- 24	M W R V R	- 20
	91	GAAAAGGGGCTACTTTGGGATTGGTCATTGCCTTATAATCGCCGCTGTCGTGGTCAAGAGTGTCAATGAGCCTAGTAATATGTCGCT	180
	- 19	KRGTYFGIWSFPLIIAAVCATQSVNDPSNNSL	11
	181	GGTTAMAGAGGGGGGGGACAGACTGTGAAAAGGCTATGACATTCGTCTGAGACCAGATTTCGGAGGTCCCCCTGTGGCAGTAGGAATGAA	270
	12	VKETVDRLLKGYDIRLRPDFGGPPVAVGMN	41
	271	CATTGATATCGCCAGCATCGATATGGTTTCTGAAGTCAATATGGACTACACCTTGACCATGTATTTCCAGCAAGCCTGGAGAGATAAGAG	360
	42	IDIA SIDN V SE V N N D Y T L T N Y F Q Q A W R D K R	71
	361 72	ACTGTECTACAATGTAATCCCTTTAAACTTGACTTGGACTAATGGAGGAGGACCAGCTCGGGGGGCGCGCGC	450 101
	451	TAAGAAGTCATTIGTACATGGAGTGACTGTCAAAAACCGTATGATTCGACTGCATCCAGATGGTACTGTCCTGTATGGCCTCAGAATCAC	540
	102	K K S F V H G V T V K N R M I R L H P D G T V L Y G L R I T	131
	541 132	AACTACAGCTGCCTGCATGGAGCTAGGGCGATCCACTGGATGAAACTGGAGATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAAGCTATGGACATCGAAGTCGAAAGCTATGGACATCGAAGTCGACATCGAAGTCGACATCGAAGTCGACATCGAAGTCGACATCGAAGCTATGGACATCGAAGTCGACATCGACGATGAAACTGGACATCGAAGTCGACATCGAAGCTATGGACATCGACGATCGAAAGCTATGGACATCGAAGCTATGGACATCGAAGCTATGGACATCGAAGCTATGGACATCGACGATCGAAAGCTATGGACATCGACGATCGAAAGCTATGGACATCGACGATCGAAAGCTATGGACATCGACGATCGAAAGCTATGGACATCGACGATCGAAGCTATGGACATCGACGATGGACATCGAAGCTATGGACATCGACGATGGAAAGCTATGGACATCGAAGCTATGGACATCGACGATGGAAAGCTATGGACATCGACGATGGACATCGAAGCTATGGACATCGACATCGACGATGGACATCGACGATGGACATCGAAGCTATGGACATCGACGATGGACATCGACGATGGACATCGAAGCTGGACATCGAAGCTATGGACATCGACGATGGACATCGACGATGGACATCGACGATGATGATGATGATGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGGACGATGATGATGATGATGATGATGATGATGATGATGATGATG	630 161
	631	TGATGACATTGAGTITTACTGGGGTGGGGATGACAATGCAGTCACGGGAGTGACAAAGATTGAGCTTCCTCAGTCTCCCATTGTAGATTA	720
	162	D D I E F Y W R G D D N A V T G V T K I E L P Q F S I V D Y	191
	721 192	TAAACTCATCAACAAAAATTGTTTTCTCCACAGGTTCTTATCCCAGATTGTCCCTAAGCTTAAGCTGAAAAAAAA	810 221
	811	CATCCTGCAGACATACATGCCATCCTGATTACCATCCTCTCCTGGGTCTCCTTTTGGATCAACTATGATGCTTCTGCTGCACGGGT	900
	222	I_L_Q_T_Y_M_P_S_I_L_I_T_I_L_S_W_V_S_F_W_I_N_Y_D_A_S_ <u>A_R_V</u>	251
	901	TGCATTAGGAATTACAACTGTCCTGACGACGACGACGATCAATACCCATGTCCGGGGAGACTCTCCCCTAAAATTCCCTATGTAAAAGCCAT	990
	252	<u>A_L_G_I_T_V_L_T_M_T_T_I_N_T_H_L</u> _R_E_T_L_P_K_I_P_Y_V_K_A_ <u>I</u>	281
	991	TGACATGTACCTAATGGGGTGCTTTGTCTTTGTCTTTATGGCCCTTCTGGAATATGCTTTGGTCAACTACATCTTCTTTGGGAAGGAGGACC	1080
	282	D_M_Y_L_M_G_C_F_V_F_N_A_L_L_E_Y_A_L_V_N_Y_L_F_F_G_R_G_P	311
	1081	CCAGCGCCAAAAGAAAAGCAGCTGAGAAAAGCTGCTAATGCCAACAACGAGAAGATGGCCCTGGATGTGCAACAAGATGGACCCACATGAGAA	1170
	312	Q R Q K K A A E K A A N A N N E K N R L D V N K <u>M</u> D <u>P M E M</u>	341
	1171	CATCTTACTCAGCACTCTTGAGATAAAAAATGAGAGGGCCACATCAGAAGCAGTAATGGGACTTGGAGACCCCAGGAGCACAATGCTTGC	1260
	342	<u>LLLSILEIKME</u> MATSEAVMGLGDPRSTMLA	371
	1261	CTATGATGCCTCCAGCATCCGGTAGCGGGAGGCGGGGTGGCCTAGGCATAGTTTTGGCCGCACGGCCTGGAACGACCATGTGGCACAAAA	1350
	372	Y D A S S I Q Y R K A G L P R H S F G R N A L E R H V A Q K	401
	1351	GAAAAGTEGEETGAGGAGGAEGTGEETECEAACTGAAATCAECEATECEEGGAETGAETGAAEGECEATTGATEGGTGGEECEGGAT	1440
	402	KSRLRRRASQLKITIPDLTDVNAIDR <u>WSRI</u>	431
	1441 432	TTTCTTCCCTGTGGTGTTTTCCTTCTTCAACATCGTCTATTGGCTTTACTATGTGAACTAAACTCCAGCCTCCCATGAGAAGCAAGGACT	1530 450
	1531 1621 1711 1801	AGATECTETI TEMAACASI TETI KARGET TEMI CECTA TA TAGASI TI GGAMACAAT CAMITA CEAGGACAAAGCASI TI TAAATI ACC TI KATI TETI CEGO TACETI TETI KATI TI TATI KATI KAGATI KATI KATI KATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI GI KAATI KAMITA KATI GA KACATI GEGA KAGATI TI TI TI GATA KATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAMAGACATI GAMAGTI KATI KAGATI GEGA KATI KATI TI TI CETI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAMAGACATI GAMAGTI KATI KAGATI GEGA KATI KATI TI TI CETI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAMAGACATI GAMAGTI KATI KAGATI GEGA KATI KATI TI TI CETI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAMAGACATI GAMAGTI KATI KAGATI GEGA KATI KATI TI TI CETI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAGATI KAMAGACATI KATI KATI KATI KATI KATI KATI KATI K	1620 1710 1800 1890
	1891	ACGCAGTTGGGACAGCACTGTGCTTATAAAACATTATCCGCAATAATCGAAACATGCTACTTCAATATGGGCTTTGAGGTCTAAGCCAGA	1980

С	1 - 25	GCAGCACCCCGGCTCGGGGTCGCGACGGCGGGGGGGGGG	90 - 19
	91 - 18	I AAGGCTTTTCGGCATCTTCTGCGCCCCGGGGCGGGGGGGG	180 12
	181	I GAAGGAGACGGTCGACAAGCTGTTGAAAGGCTACGACATTGGCTGAGGGGATCGAGGGGGCCCCCCAGTCTGCGTGGGGATGAACAT	270
	13	5 K E T V D K L L K G Y D I R L R P D F G G P P V C V G M N I	42
	271	I CGACATCGCCAGCATCGACATGGTTTCTGAAGTCAACATGGATTATACCTTAACTATGTATTTCCAACAATATTGGAGAGATAAAAGGCT	360
	43	5 D I A S I D H V S E V N N D Y T L T H Y F Q Q Y W R D K R L	72
	361 73	I CGCCTACTCTGGGATCCCTCTAACCCTCACGCTGACAATGGACTGGCGGCGCGGGCGG	450 102
	451	1 AAAGTCATTIGTGCACGGAGTGACAGTGAAAAACCGCATGATCCGCCTCCACCCTGATGGAACAGTGCTGTACGGGCTCAGGATCACCAC	540
	103	3 k s f v h g v t v k n r m i r l n p d g t v l y g l r i t t	132
	541	1 CACAGCAGCTTGCATGATGAGCCTCAGAAGATACCCACTGGATGAGCAAAACTGCACCCCTGGAAATTGAAAGCTATGGATACACCACGGA	630
	133	5 T A A C M M D L R R Y P L D E Q C T L E I E S Y G Y T T D	162
	631	1 TGACATIGAATITTACIGGCGTGGCGGGGGAAAGGCIGTIACIGGCGGGGAAAGGAICGAGCICCCACAGIICICCAITGIGGAGCACCG	720
	163	3 D I E F Y W R G G D K A V T G V E R I E L P Q F S I V E H R	192
	721	I TETGGTETECAGGAATGTTGTETTEGEEACAGGTGECTACCETEGAATGAGTTTEGGTTGAGGAGAAACATIGGGTACTTCAT	810
	193	3 L V S R N V V F A T G A Y P R L S L S F R L K R N I G <u>Y F I</u>	222
	811 223	1 ACTTCAGACGTATATGCCCTCAATCATGATCATCCTCCTCAGGGTGTCCTTCTGGATCAATTATGATGCATCTGCTGCCGCGCGCG	900 252
	901	1 CCTAGGGATTACCACCGTGCTCACCATGACAACCATCAACACTACCTTCGAGAGACTCTACCCCAAAATCCCCTAAGTCAAAGCCATCGA	990
	253	3 <u>L G I T T V L T M T T I N T H L</u> R E T L P K I P Y V K A <u>L D</u>	282
	991	1 CATGTACCTGATGGGTTGCTTCGTCTTGTATTCCTGGCACTATGCCTTTGTCAACTATATTTTCTTTGGACGAGGTCCCCA	1080
	283	3 <u>M Y L M G C F V F V F L A L L E Y A F V</u> N Y I F F G R G P O	312
	1081	1 ACGGCAGAAGAAGCTTGCGGAGAAGACCAGGCCAAGGCCAAGAATGATCGATGCAGGGAAAATCAACCGGGTGGATGCTCACGGGAATAT	1170
	313	3 R Q K K L A E K T A K A K N D R S K S E I N R V D A H G N I	342
	1171	1 CCTACTAGCACCGATGGATGTTCACAATGAAATGAAGATGAGGTTGCAGGCGGCGTTGGTGACACCAGGAATTCAGCAATATCCTTTGACAA	1260
	343	3 L L A P M D V H N E M N E V A G S V G D T R N S A I S F D N	372
	1261	1 CTCAGGAATCCAGTATAGGAAACAGAGCATGGCCAAGGAGGCGGGGGAGATGGAGGAGGAAGCATCCCGGACAAGAAGCGCA	1350
	373	3 S G I Q Y R K Q S M P K E G H G R Y M G D R S I P H K K T H	402
	1351	1 CCTACGGAGGAGGTCTTCGCAGCTCAAAATCAAAATCCCTGATCTAACCGATGTGAATGCCATAGACAGATGGTCCCGGATCGTGTTTCC	1440
	403	3 L R R R S S Q L K I K I P D L T D V N A I D R <u>U S R I V F P</u>	432
	1441	1 ATTCACCTTTTCTCTTTCACTTAGTTTACTGGCTGTACTGTTACTGAGTGACTGTACTGATT);;TCAAAGACTTCATTTAACA	1530
	433	3 <u>F T F S L F N L V Y M L Y Y</u> N	448
	1531 1621 1711 1801	CIGAGIGAANIATIACCCCICCCIGCTGCAGITITIATACCAGIATACACACACACACACACACACACACACACACACACACA	1620 1710 1800 1890
	1891 1981	1 GCCTCAAGAATGAGGGGGAGAAACACAGTCATCCCAAAGTGTGTCTTTTATTATCATAAGTTTTTGCTTAAGAATCAAAACGGAATTCTT 1 Agytaatctttgggcactcc 2000	1980

Fig. 1. Nucleotide and deduced amino acid sequences of rat GABA_A receptor $\beta 1$ (a), $\beta 2$ (b) and $\beta 3$ (c) subunits. Nucleotide and amino acid positions are indicated. Negative numbers refer to signal peptide residues. Potential N-linked glycosylation sites carry asterisks, the β -structural loop flanked by cysteines is indicated by a broken line and the four transmembrane regions are underlined. Putative regulatory sites in the intracellular region of the β subunits are denoted by small circles. Underlined residues in $\beta 2$ (positions 336–352) correspond to a chemically determined peptide sequence of the homologous bovine subunit.

Importantly, in both the rat and bovine $\beta 2$ subunits this domain contains one of the peptide sequences (MXPHENI-LLSTLEIKNE) chemically determined from cyanogen bromide-cleaved affinity-purified bovine GABA_A receptor (Schofield *et al.*, 1987). The $\beta 2$ subunit sequence is the only one in which a methionine residue (the site of cyanogen bromide cleavage) precedes this peptide sequence (Figures 1 and 2) and residues P₃, E₅ and K₁₅ uniquely distinguish the $\beta 2$ from the $\beta 1$ and $\beta 3$ sequences. This demonstrates that the $\beta 2$ polypeptide is a component of the natural GABA_A receptor complex. Interspecies homologies for β subunits are extremely high since on average only 10 residues are

25 25 - 24 22 - 25 - 25	$ \begin{array}{c} M \forall : I V O N K \neq V I G I I S F F V M V A M V C C A H S S N F F S N N N V V K K N N V V K K N N V V K K N N V V K K N N V V V K K N V V V K K V V V V K V $	RATBETAL BOVBETAL RATBETA2 BOVBETA2 RATBETA3 BOVBETA3
16 16 16 16 16	V D K I L K G Y D I K I R P D F G G P P V D V G M R I D V A S I D M V S E V N K V D K I L K G Y D I K I R P D F G G P V D V G M K I D V A S I D M V S E V N K V D K I L K G Y D I R I R P D F G G P V A V G M N I D I A S I D M V S E V N K V D K I L K G Y D I R I R P D F G G P V V G M N I D I A S I D M V S E V N M V D A I L K G Y D I R I R P D F G G P V V C M N I D I A S I D M V S E V N M V D A I L L K G Y D I R I R P D F G G P V V C W N I D I A S I D M V S E V N M V D A I L L K G Y D I R I R P D F G G P P V C V G M N I D I A S I D M V S E V N M	RATBETAL BOVBETAL RATBETA2 BOVBETA2 RATBETA3 BOVBETA3
56 56 56 56	DYTITMYFQQYWKDKRISYGGIPINLTLDNRVADQLWYFD DYTITMYFQGYWKDKRISYNGIPINLTLDNRVADQLWYFD DYTITMYFQQAWDKRISYNNYFPINLTIDNRVADQLWYFD DYTITMYFQAWBDKRISYNNYFPINLTIDNRVADQLWYFD DYTLTMYFQQYWRDKRLAYSGIPINLTLDNRVADQLWYFD DYTLTMYFQQYWRDKRLAYSGIPINLTLDNRVADQLWYFD	RATBETA1 BOVBI FA1 RATBI FA2 BOVBE FA2 RATBETA3 BOVBETA3
96 96 93 93 96 96	TYFLNDERSFVHGVTVENEMIRLHPDGTVLYGLRITTTA TYFLNDERSFVHGVTVENEMIRLHPDGTVLYGLRITTTA TYFLNDERSFVHGVTVENEMIRLHPDGTVLYGLRITTTA TYFLNDERSFVHGVTVENEMIRLHPDGTVLYGLRITTTA TYFLNDERSFVHGVTVENEMIRLHPDGTVLYGLRITTTA TYFLNDERSFVHGVTVENEMIRLHPDGTVLYGLRITTA	RATBETA1 BOVBETA1 RATBE1A2 BOVBE1A2 RATBETA3 BOVBETA3
136 136 136 136 136 136		RATBETAL BOVBETAL RATBETA2 BOVBETA2 RATBETA3 BOVBETA3
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Fig. 2. Comparison of three rat and bovine GABA_A receptor β subunits. Numbering and symbols are as in Figure 1.

substituted between the rat and bovine homologues. A similarly high degree of sequence conservation has been observed for the bovine (Schofield *et al.*, 1987) and human (Schofield *et al.*, 1989) $\alpha 1$ and $\beta 1$ subunits.

β subunit localization

The extent of β subunit expression in the brain was investigated by Northern blot analysis. RNA samples were prepared from rat and calf total brain as well as from the cortex, hippocampus and cerebellum. Northern blots of these RNAs were hybridized with ³²P-labelled oligonucleotides complementary to DNA sequences encoding the divergent intracellular domain of the three β subunits. The autoradiographs (Figure 3) document that in the rat and bovine brain both the β 2 and β 3 subunits are considerably more abundant than the β 1 subunit. The relative abundance of the β subunit mRNAs parallels that of the respective cDNA clones in the brain libraries.

In rat brain, the hippocampus and cerebellum contain the highest levels of β subunit mRNAs while the cortex shows only small amounts of β -specific RNA. β 2 subunit mRNA is altogether more abundant than β 3 mRNA. The three β subunit encoding mRNAs differ in size, with β 1 mRNA being the largest (~12 kb) followed by β 2 mRNA (~8 kb) and β 3 mRNA, which is represented by two size forms



Fig. 3. Regional brain distribution of β subunit mRNAs. Northern blots of poly(A)⁺ RNA from rat (A) and calf (B) cortex (Cx), hippocampus (Hi), cerebellum (Cb) and total brain (Br) were probed using β 1, β 2 and β 3 subunit-specific oligonucleotides. Size markers indicated on the left represent 9.5, 7.5, 4.4 and 2.4 kb.



Fig. 4. Expression of $GABA_A$ receptor β subunits in *Xenopus* oocytes. Clamp potential was -70 mV. Downward deflections reflect inward currents (see calibration bar). GABA-application (1 and 3 μ M for each subunit pair) is indicated by horizontal bars.

(~6 kb and ~2.5 kb), the smaller transcript being the major form in the rat cerebellum.

As observed for the α subunit mRNAs (M.Köhler and P.H.Seeburg, unpublished), the bovine brain contains substantially higher amounts of β subunit mRNA than the rat brain. In particular, the cortex and cerebellum display high levels of $\beta 2$ and $\beta 3$ mRNAs. Two $\beta 1$ mRNAs (13 and 4 kb) are observed which are of equal abundance in calf cortex, whereas the smaller transcript appears to be the major form in cerebellum; $\beta 2$ mRNA exists as 8 kb (major form) and 7 kb transcripts in cortex and cerebellum. Several transcript sizes originating from one gene have been observed previously and usually reflect the use of different polyadenylation sites to generate different-sized 3' flanking regions (Setzer *et al.*, 1980; Tosi *et al.*, 1981).

Three β subunits in the adrenal medulla

Determination of the in vivo subunit composition of a single type of GABA_A receptor may help to resolve the role of multiple α and β subunit polypeptides. The adrenal medullary chromaffin cells express electrophysiologically (Bormann and Clapham, 1985) and pharmacologically (Kataoka et al., 1984) well-characterized GABAA receptors that might constitute a single population of receptors. To analyse these receptors in molecular terms we constructed a bovine adrenal medulla cDNA library and screened it at low stringency using as probes ³²P-labelled $\alpha 2$ (Levitan et al., 1988) and β 1 (Schofield et al., 1987) subunit cDNAs as well as the degenerate oligonucleotide based on the conserved octameric peptide sequence in M2 (see above). Clones encoding $\alpha 1$, $\beta 1$, $\beta 2$ and $\beta 3$ subunits were obtained. but no $\alpha 2$ or $\alpha 3$ subunit cDNAs were found. While these results do not prove the absence of α variants, they suggest that these subunits represent at best very minor species in the adrenal medulla. This is substantiated by the absence of α^2 and α^3 subunit specific hybridization signals on Northern blots of adrenal medullary RNA (not shown). However, the presence of three β subunits may indicate that chromaffin $GABA_A$ receptors constitute a heterogeneous population.

All adrenal cDNA sequences were colinear with those of brain-derived cDNAs. Occasionally observed third-base substitutions in codons are probably a consequence of different RNA sources used in cDNA library construction. These results show that many of the genes encoding $GABA_A$ receptor subunits are expressed both centrally and peripherally without the use of alternative splicing. Hence, alternate exon usage is not a major mechanism for generating diversity of $GABA_A$ receptors.

Expression in Xenopus oocytes

We investigated whether the novel β subunits could contribute to the formation of functional GABA_A receptors by expressing each rat β subunit in combination with the rat α 1 subunit (unpublished) in *Xenopus* oocytes. Expression was achieved by nuclear injection (Voellmy and Rungger, 1982) of pairs of recombinant CDM8 vectors (Seed, 1987) in which the cloned cDNAs are under the transcriptional control of the cytomegalovirus promoter. This mode of expression is sensitive to small amounts of DNA and circumvents the need for the *in vitro* synthesis of RNA (Ballivet *et al.*, 1988). Electrophysiological recordings (Figure 4) showed that either the β 2 or β 3 polypeptide can be substituted for the β 1 subunit to yield dose-dependent GABA-evoked currents. These were always inhibited by bicuculline, enhanced by the barbiturate pentobarbital and had reversal potentials (E_r) close to the chloride equilibrium potential ($E_r - 23 \text{ mV}$) of *Xenopus* oocytes (Dascal *et al.*, 1984), indicating that each receptor forms GABA-gated chloride channels (not shown). Thus, substitution of either the $\beta 2$ or $\beta 3$ subunit for the $\beta 1$ subunit does not appear to change the qualitative properties of the expressed GABA_A receptors.

Discussion

Molecular cloning has revealed heterogeneity of the GABA_A receptor β subunit. The assignment of the two novel subunits $\beta 2$ and $\beta 3$ as true components of the GABA_A receptor is based on the observations that (i) β 1, β 2 and β 3 subunits share a high degree of sequence identity, (ii) the $\beta 2$ subunit contains a peptide sequence obtained by chemical means from affinity-purified GABAA receptor complex and (iii) all three subunits form GABA-responsive chloride channels when co-expressed with the $\alpha 1$ subunit. The β subunits are highly sequence-conserved and contain a larger intracellular domain than other subunits (Levitan et al., 1988; Pritchett et al., 1989). This domain in the β subunits contains a consensus site for cAMP-dependent phosphorylation by protein kinase A (Feramisco et al., 1980). The presence of this site strengthens our earlier hypothesis (Schofield et al., 1987) that the β subunit provides a target for the cellular regulation of GABA_A receptor activity.

While molecular heterogeneity of the α subunit was anticipated, the existence of β subunit variants is unexpected. Northern analysis indicates that the novel β subunits far exceed the β 1 subunit in abundance. This result provides an explanation for the previously noted imbalance of *in situ* hybridization signals for α and β subunit mRNA in regions of rat and bovine brain (Séquier *et al.*, 1988; Siegel, 1988). In fact, evidence from this laboratory indicates that the different β subunits are indeed expressed in distinct, possibly overlapping neuronal populations (B.D.Shivers, unpublished).

The increasing number of variants of GABA_A receptor α and β subunits poses the problem of determining the subunit composition of natural GABA_A receptors. As the analysis of a homogeneous GABA_A receptor population may provide one way of dissecting the subunit composition, we investigated the receptor in adrenal medullary chromaffin cells by molecular cloning. Besides finding only one α subunit we were surprised by the presence of all three β subunits. Thus, either all β variants are part of the same receptor or the adrenal chromaffin GABAA receptors are composed of different subtypes. Of these alternatives, the first is less likely considering the different expression levels of the three β subunit mRNAs and their distinct localization in the brain (unpublished). While GABAAreceptor diversity in chromaffin cells has yet to be substantiated by electrophysiology or pharmacology, the presence of several conductance states in the GABAA receptor of adrenal chromaffin cells (Bormann and Clapham, 1985) might indicate such a heterogeneity. It will be of interest to examine other peripheral GABA_A receptor populations, such as those involved in the control of pituitary hormone release (Grandison and Guidotti, 1979; Racagni et al., 1979).

The role of the β subunits in generating different GABA_A

receptor subtypes remains to be established. Combinations of different α and β subunits may define receptors that can be distinguished by pharmacology and channel properties, generating a greater diversity of GABA responses than would be achieved with fewer receptor subtypes. While the true extent of α and β subunit heterogeneity is unknown, recent cDNA cloning experiments in this laboratory have provided evidence for the existence of additional GABA_A receptor α subunits and of novel subunits not of the α or β type (Pritchett *et al.*, 1989). However, no additional β subunit variants were identified.

Materials and methods

Isolation of cDNA clones

A λ gt10 bovine brain cDNA library (Schofield *et al.*, 1987) was screened with a 96-fold degenerate 32 P-labelled 23mer oligonucleotide encoding a conserved octameric peptide sequence in M2 of GABAA receptor subunits, 5' AC(A,C)AC(A,T)GT(G,T)CT(A,C,G)AC(A,C)ATGAC(A,C)AC 3'. Only indicated third position choices were included. Known subunits were identified using $\alpha 1$, $\alpha 2$, $\alpha 3$ and β subunit-specific oligonucleotides (Levitan et al., 1988; and see below). cDNAs hybridizing to the 23mer but not to the subunit-specific oligonucleotides were sequenced in λ g10 or after subcloning into M13 vectors (Vieira and Messing, 1987) by the chain termination method (Sanger et al., 1977). Sequencing reactions using the 23mer oligonucleotide were performed with 0.5 μM primer and reactions were at 55°C when recombinant λ DNA was used as template. A rat forebrain cDNA library was screened with the following ³²P-labelled β subunitspecific oligonucleotides complementary to sequences encoding part of the large intracellular domain of the three subunits: β 1; 75mer (5' TČCCACGCCCGTGAGCACTTCAGAGCCGCTCGTCTCGTTCCT-GATCTCCAGGGTACTGAGGAGAATGTTGCCGTG 3'); β 2, 60mer (5' TTTCCGATACTGGATGCTGGAGGCATCATAGGCCAGCATTGT-GCTCCTTGGGTCTCCAAG 3'); β 3, 60mer (5' TCTTGCTGAATTC-CGGGTATCACCAACGCCGCCGGCAACCTCGTTCATCTCATTG-TGAAC 3'). The longest cDNA clones were completely sequenced. Furthermore, a bovine adrenal medulla cDNA library was constructed in λgt10 by standard methods (Huynh et al., 1985) and screened using as probes both the degenerate 23mer oligonucleotide and two internally labelled EcoRI fragments of cloned bovine $\beta 1$ and $\alpha 2$ subunit encoding cDNAs. The $\beta 1$ cDNA fragment comprised nucleotides 1-726 (Schofield et al., 1987) and the α^2 cDNA fragment contained nucleotides 133-1755 (Levitan et al., 1988). Sequence analysis was as described above.

Northern blot analysis

RNA was isolated by published methods (Chomczynski and Sacchi, 1987) from three brain regions of 8-month-old calf and young adult rats (200 g). Poly(A)⁺ RNA was prepared using oligo(dT) – cellulose chromatography. For Northern analysis, RNA (3 μ g) was electrophoresed in 1.2% formaldehyde-containing agarose gels and blotted onto nitrocellulose. These blots were hybridized to subunit-specific ³²P-labelled (sp. act. 10⁶ c.p.m./pmol) oligonucleotides in 40% formamide at 42°C, washed in 2 × SSC, 0.1% SDS at 55°C and exposed to X-ray film, using an intensifying screen at -80°C for 5 days (bovine) or 14 days (rat). For the bovine Northerns the oligonucleotide sequences are listed above. For the rat experiment, the β 1and β 2-specific oligonucleotides were 5' GTAAGAGAGAAGCCCCAA ACTCACTTAGTCTGTCTGCGATTTTGTACTGTC 3' and 5' AGAGA-GGAGATCCACCCAGTGCAGTAATTC 3', while the same β 3 probe was used.

Expression in Xenopus oocytes

The rat $\alpha 1$ (unpublished), $\beta 1$, $\beta 2$ and $\beta 3$ subunit-encoding cDNAs were cloned into the CDM8 vector (Invitrogen, San Diego, CA). Either *XhoI* or *Eco*RI fragments containing the entire coding sequences were converted to blunt ends and ligated with adaptor sequences to generate *BstXI* cohesive termini. The adaptor sequences were 5' CGAATTCAGAGAACA 3' and 5' CTCTGAATTCG 3'. The terminally modified cDNAs were then used to replace the stuffer fragment in CDM8 (Seed, 1987). Orientations of subcloned cDNAs relative to the vector-carried cytomegalovirus promoter were determined by restriction analysis. The nuclei of occytes were injected (Voellmy and Rungger, 1982) with these constructs (10 nl, 350 pg of each expression plasmid). After incubation of injected oocytes at 19°C for 3-6 days, currents were recorded in a conventional two-electrode voltage clamp in normal frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM

CaCl₂, 10 mM Hepes, pH 7.2) before and during superfusion with frog Ringer containing different concentrations of GABA. Bicuculline was used at 10 μ M and pentobarbital at 5 μ M.

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