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The third γ subunit of the γ-aminobutyric acid type A receptor family

(benzodiazepine receptor/cDNA cloning/electrophysiology/*in situ* hybridization)

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ABSTRACT **Cloned cDNAs encoding a member of the γ-aminobutyric acid type A receptor γ-subunit class were isolated from rat-brain-mRNA-derived libraries. The γ_3 mRNA is present in cortex, claustrum, caudate putamen, and some thalamic nuclei, particularly the medial geniculate nucleus, where it is the predominant γ-subunit transcript. The γ_3 gene is expressed at very low levels in cerebellum and hippocampus. In coexpression experiments with the α_1 and β_2 subunits, γ_3 imparts benzodiazepine binding to γ-aminobutyric acid type A receptors and forms γ-aminobutyric acid-gated benzodiazepine-modulated chloride channels that exhibit a larger conductance than $\alpha_1\beta_2$ receptor channels. Furthermore, the presence of γ_3 in place of γ_2 in $\alpha_1\beta_2\gamma_x$ receptors generates a marked decrease in the affinity of agonists while leaving the affinity of antagonists or negative modulators largely unaffected.**

γ-Aminobutyric acid type A ($GABA_A$) receptors consist of subunits whose assembly forms a neurotransmitter-gated anion channel. Subunits for this receptor constitute a large family whose members are classified according to primary structure as α, β, γ, δ, and ρ subunits (1–3). Recombinant expression studies of different α variants in combination with a β variant and the γ_2 subunit demonstrate that $GABA_A$ receptors with distinct pharmacological properties are generated (2, 4–6). A distinctive mark of these $\alpha_x\beta_x\gamma_2$ receptors is their ability to bind modulatory compounds such as benzodiazepines (BZs), which can modulate γ-aminobutyric acid (GABA)-gated channel activity at an allosteric site. The α variants determine the affinity of $GABA_A$ receptor subtypes toward these modulatory compounds, and members of the γ-subunit class, in particular the γ_2 variant, are essential for the architecture of the BZ site (7). Furthermore, the γ subunits impart a large unitary conductance on $GABA_A$ channels (8–10).

The γ-subunit family (7, 10, 11) appears to be less diverse than the α-subunit gene family, which has six members (2, 3, 12). In a search for further γ subunits, we isolated and sequenced a third γ variant (γ_3) (Fig. 1; §) and found that its presence in recombinant $GABA_A$ receptors can markedly change the interaction of compounds specific for the BZ site.

MATERIALS AND METHODS

Cloning and Expression of Rat γ_3-Subunit cDNA. A rat forebrain cDNA library constructed in λZAPII (Stratagene) was screened with a rat γ_2 cDNA fragment containing the coding region for γ_2, residues 343–429 (11). Several isolates were found to encode an additional γ subunit, γ_3, and a full-length cDNA was sequenced. For expression, this cDNA was subcloned into the pCIS2 vector (13) and this expression construct was used in concert with those for rat α_1 and β_2 for 293 cell transfection (5, 14).

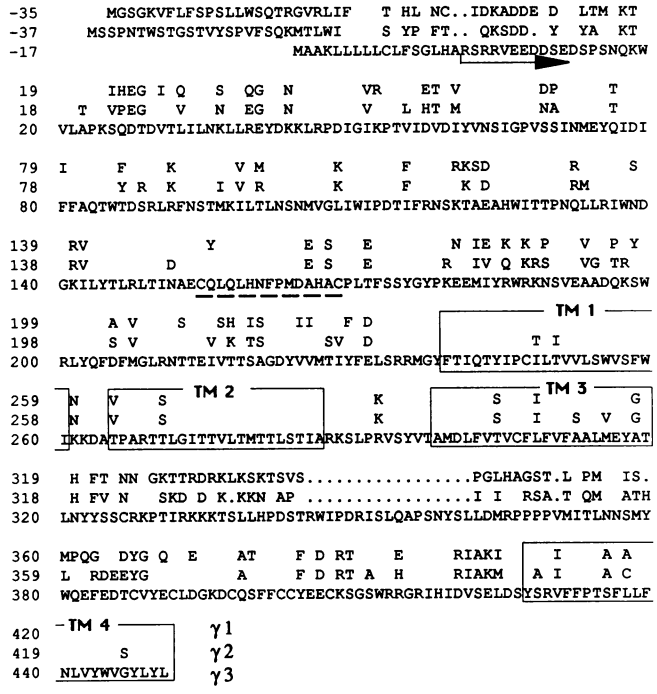

FIG. 1. Comparison of primary structures of rat γ_1, γ_2, and γ_3 $GABA_A$ receptor subunits (top, middle, and bottom sequences, respectively). Sequences are numbered on the left and only substitutions in γ_1 and γ_2 relative to γ_3 are listed. Transmembrane (TM) regions are boxed. Note the insertion in γ_3 relative to the γ_1 and γ_2 polypeptides. Arrow, start of mature polypeptide; dashed underline, Cys–Cys loop.

Membrane Preparation and Ligand Binding. Experiments were performed as described (5). The K_d for [^{3}H]Ro 15-1788 was determined using a concentration range of 0.2 nM–20 nM. IC_{50} values for diazepam and flunitrazepam were determined in concentration ranges of 0.2–30 μM and 0.05–10 μM, respectively. Data were analyzed by nonlinear regression and IC_{50} values were converted to K_i values by the Cheng–Prusoff equation (see Table 2).

Electrophysiology. Transfected 293 cells were studied with the single-electrode voltage clamp technique in the whole-cell

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Abbreviations: BZ, benzodiazepine; GABA, γ-aminobutyric acid; $GABA_A$ receptor, γ-aminobutyric acid type A receptor.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M81142).

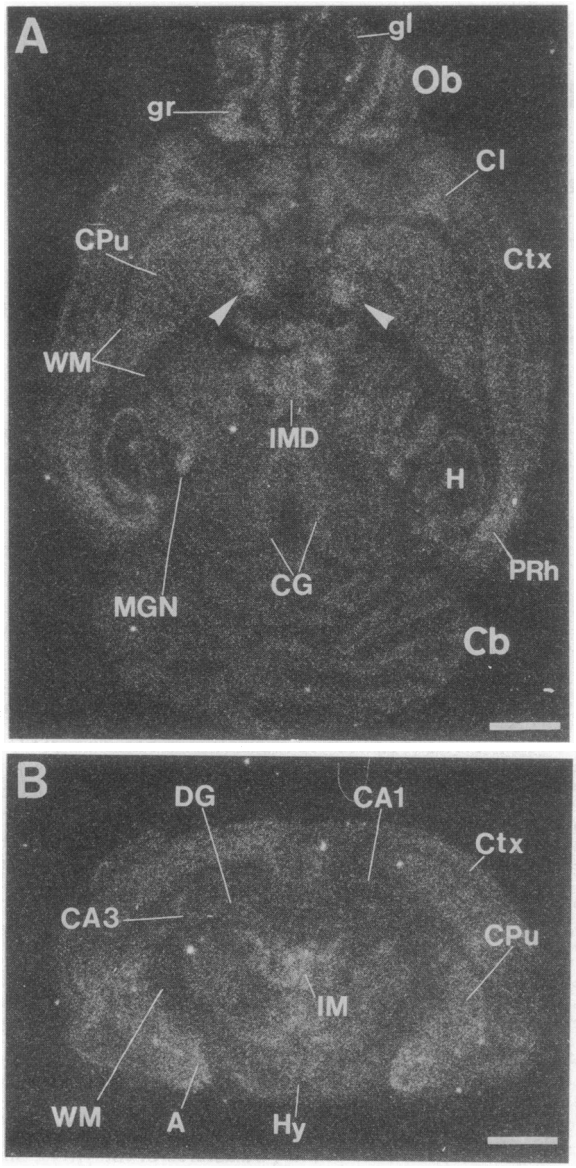

FIG. 2. Distribution of γ_3 mRNA in horizontal (*A*) and coronal (*B*) sections of adult rat brain. A, amygdala; Cb, cerebellum; CG, central grey; Cl, claustrum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; gl, glomerular layer of olfactory bulb; gr, granule cell layer of olfactory bulb; H, hippocampal formation; Hy, hypothalamus; IMD, intermediodorsal thalamic nucleus; MGN, medial geniculate thalamic nucleus; Ob, olfactory bulb; PRh, perirhinal cortex; WM, white matter tracts. Arrowheads in *A* tentatively designate septohypothalamic nuclei. Exposure time was 4 weeks to Kodak XAR-5 film. (Bars: *A*, 2.75 mm; *B*, 2.5 mm.)

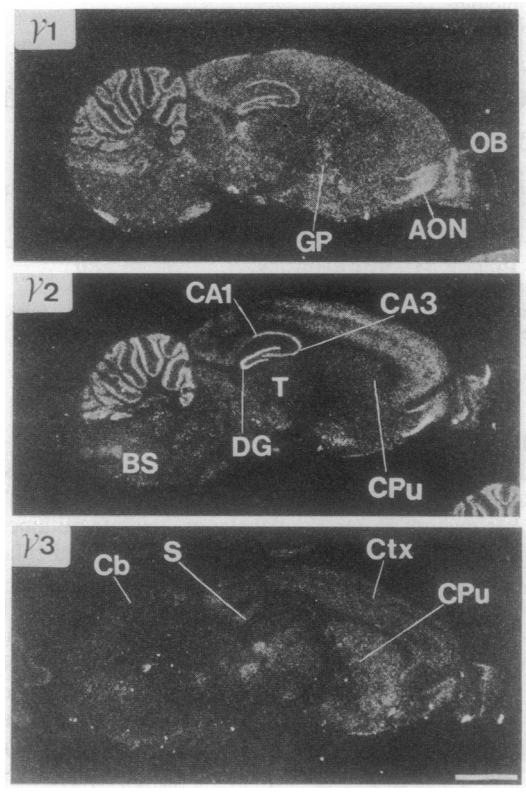

FIG. 3. Distribution of three γ-subunit mRNAs in parasaggital adult rat brain sections. AON, anterior olfactory nucleus; BS, brain stem; Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; GP, globus pallidus; OB, olfactory bulb; S, subiculum; T, thalamus. All images result from the same exposure time (3 weeks) and are designed to show relative differences in γ signal intensity. (Bar = 4 mm.)

configuration (15) as detailed in ref. 16. Drugs were applied for 5 sec between two GABA (1 μM) pulses delivered every 10 sec. The maximal Cl^- current measured from each cell was larger (>1 nA) than the test response of 150–200 pA that we used, indicating that the percentages of potentiation we observed were far below the maximal efficacy of the system. Unitary currents activated by GABA (1 μM) were studied in outside-out excised patches from expressing cells.

***In situ* Hybridization.** Two γ_3 oligonucleotides constructed complementary to amino acids 343–358 (5′-AGAGGGTGCTTGAAGGCTTATTCGATCAGGAATCCATCTTGTTGA-3′) and amino acids 357–371 (5′-CATCACGGGTGGTGGGGGTCTCATATCCAGGAGAGAATAATTAGA-3′) gave identical patterns. The γ_1 and γ_2 oligonucleotides were as in refs. 10 and 11. Procedures are described in ref. 17 and anatomical assignments were according to Paxinos and Watson (18).

RESULTS

The Primary Structure of γ_3. The DNA sequence of γ_3 cDNA isolates from a rat forebrain cDNA library predicts an open reading frame of 1401 nucleotides encoding a polypeptide of 467 residues with all the sequence features of a $GABA_A$ receptor subunit. A particularly close sequence correspondence of this polypeptide was seen with the γ_1 (10) and γ_2 (11) subunits (Fig. 1). Whereas the sequences of γ_1 and γ_2 are 71.4% identical, γ_3 shows 64.6% identity with γ_2 and 63.8% with γ_1. The slightly reduced sequence identity of γ_3 with the other γ variants is due to a unique insertion of 17 residues in the cytoplasmic domain of γ_3 relative to the γ_1 and γ_2 polypeptides. This insertion sequence in γ_3 does not specify consensus phosphorylation sites and, from PCR analysis (A.H. and P.H.S., unpublished data), does not appear to be the product of alternative splicing, which characterizes a longer form of the γ_2 subunit (19–21).

Expression in Brain. We examined the sites of expression of the γ_3 gene by using *in situ* hybridization (Figs. 2–4). The γ_3 mRNA appears to be considerably scarcer in rat brain than γ_1 and γ_2 mRNAs and, consequently, γ_3 autoradiographs required long exposure times. An obvious feature of the γ_3 mRNA distribution, when examined in horizontal and parasaggital sections, is the scarcity of signal in cerebellum and hippocampus (Figs. 2 and 3). These latter two regions contain appreciable quantities of γ_2 mRNA and, to some extent, γ_1 transcripts (Fig. 3). The most notable regions for the occurrence of γ_3 mRNA are olfactory bulb (granule cell

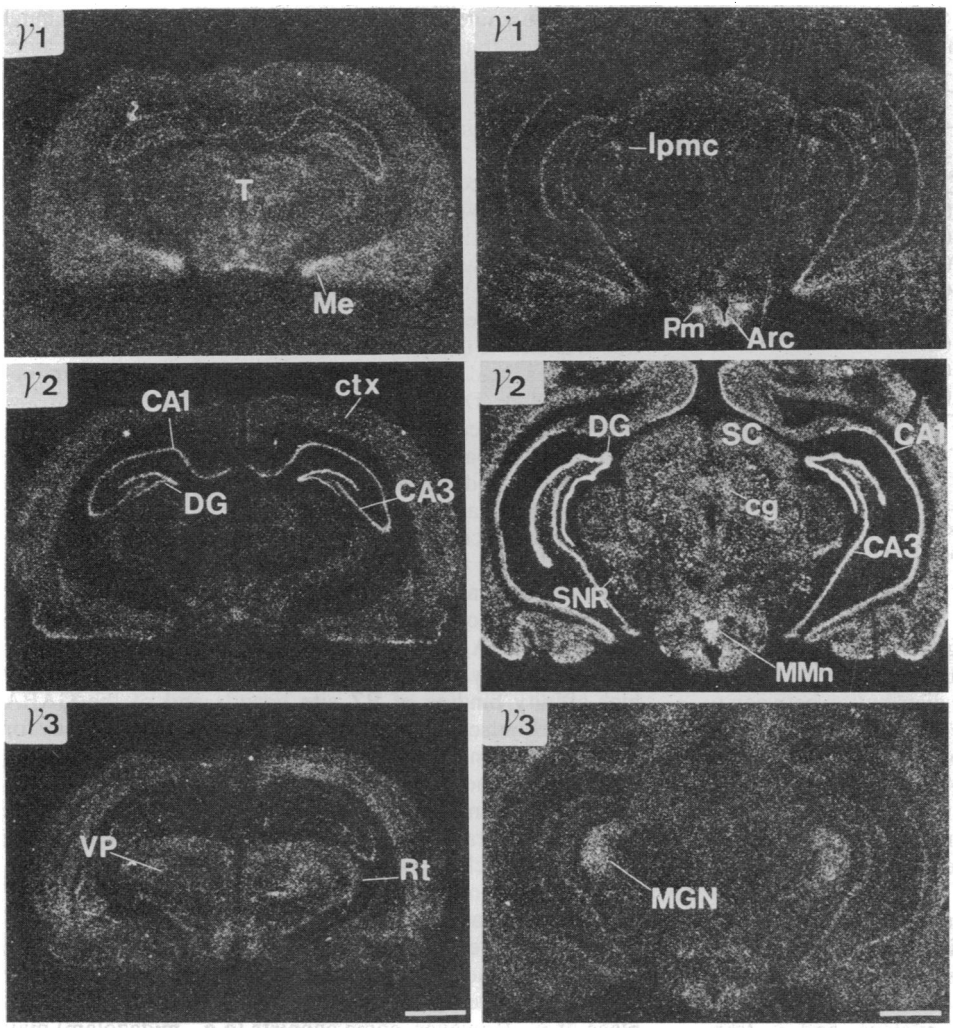

FIG. 4. Distribution of three γ-subunit mRNAs in coronal adult rat brain sections. (*Left*) More rostral structures. (*Right*) Same structures at a higher enlargement factor. All sections were taken at the level of the superior colliculus. Arc, arcuate nucleus; ctx, cortex; cg, central grey; DG, dentate gyrus; lpmc, lateral posterior thalamic nucleus, mediocaudal; MGN, medial geniculate nucleus; MMn, medial mammillary nucleus; Pm, premammillary nucleus; Rt, reticular thalamus; sc, superior colliculus; SNR, substantia nigra reticulata; T, thalamus; VP, ventral posterior nucleus. Exposure times were as for Fig. 2. (Bars: *Left*, 2.7 mm; *Right*, 1.6 mm.)

and glomerular layers) cortex, caudate putamen (striatum), and some thalamic nuclei. Among the three γ-subunit mRNAs present in the caudate putamen, γ_3 mRNA appears to be marginally the most prevalent (Figs. 2 and 3). This mRNA is also the most abundant γ-subunit transcript in the nucleus accumbens (data not shown) and the claustrum (Fig. 2).

The γ_3 gene is also expressed relatively highly in a pair of nuclei adjacent to the medial caudate, these being tentatively identified as the septohypothalamic nuclei (Fig. 2). The relative exclusion of γ_3 mRNA from hippocampus and its expression in cortex and thalamus are further emphasized when examining coronal autoradiographs (Figs. 2 and 4). All three γ-subunit mRNAs are present at low levels in many

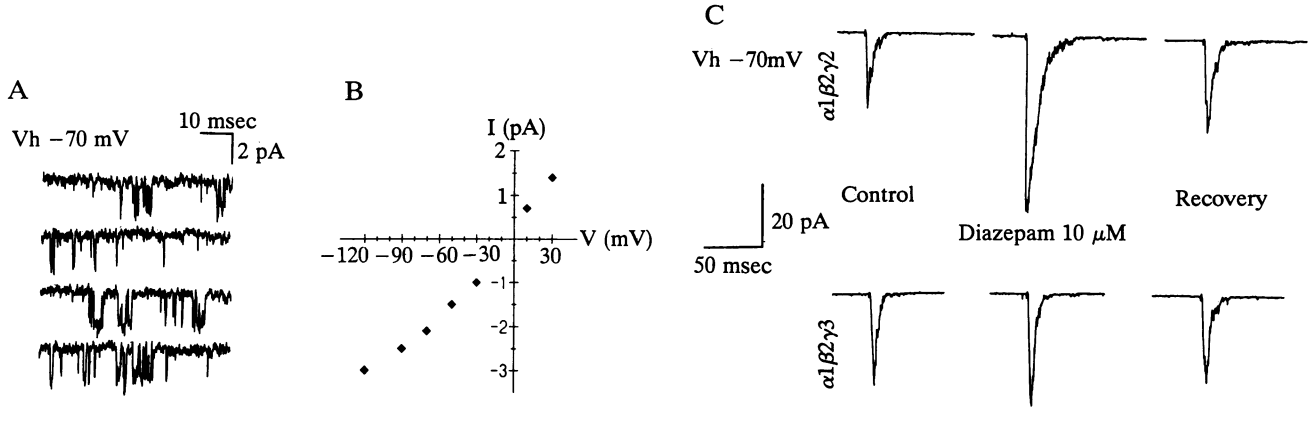

FIG. 5. Electrophysiological properties of $\alpha_1\beta_2\gamma_3$ receptors. (*A*) Single-channel currents activated by GABA (1 μM) in an outside-out patch excised from 293 cells engineered to express $\alpha_1\beta_2\gamma_3$ receptors. (*B*) Current (I)–voltage (V) relationship for the channel current amplitude in the same patch. (*C*) Diazepam potentiates Cl^- currents elicited by iontophoretical application of GABA on a multichannel outside-out membrane patch excised from 293 cells expressing $\alpha_1\beta_2\gamma_2$ (upper traces) and $\alpha_1\beta_2\gamma_3$ (lower traces) receptors. Vh, holding voltage.

Table 1. BZ receptor ligands modulate GABA-activated Cl^- currents through recombinant $\alpha_1\beta_2\gamma_3$ and $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor channels

Drug	Cl^- current, % change	
	$\alpha_1\beta_2\gamma_3$ channels	$\alpha_1\beta_2\gamma_2$ channels
Flunitrazepam (10 μM)	75 ± 12 (5)	104 ± 16 (7)
Diazepam (10 μM)	25 ± 6 (5)	122 ± 20 (8)
Zolpidem (10 μM)	20 ± 3 (4)	250 ± 50 (4)
DMCM (10 μM)	−66 ± 6 (5)	−63 ± 9 (3)
DMCM (10 μM) + flumazenil (10 μM)	2 ± 3 (2)	0 ± 5 (3)
$ZnCl_2$ (10 μM)	−21 ± 2 (4)	−9 ± 8 (10)*
$ZnCl_2$ (100 μM)	−43 ± 7 (3)	−17 ± 16 (9)*

Whole-cell GABA (1 μM)-activated Cl^- currents were recorded by voltage-clamp at a holding potential of −50 mV from 293 cells expressing $\alpha_1\beta_2\gamma_2$ or $\alpha_1\beta_2\gamma_3$ receptors. Each value (mean ± SEM) represents the percent increase (decrease in negative numbers) relative to the control obtained from the reported number of cells (in parentheses). DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate.
*Values taken from ref. 23.

thalamic nuclei compared to often significant thalamic levels of α_1, α_4, β_2, and δ transcripts (11, 12, 22). However, there are certain differences in the thalamic distribution between γ_2 and γ_3 mRNAs. The most discernable difference occurs in the medial geniculate nucleus, which expresses higher quantities of γ_3 mRNA than of γ_1 and γ_2 mRNAs (Fig. 4). The γ_3 gene expression is also elevated in the intermedial dorsal and associated thalamic nuclei (Fig. 2). In contrast, γ_1 mRNA is restricted to a small nucleus on the dorsal surface of the medial geniculate nucleus (Fig. 4).

As noted (10), the γ_1 mRNA predominates in certain amygdaloid nuclei (medial amygdaloid nucleus), some hypothalamic nuclei (e.g., arcuate nucleus), and the premammillary nucleus (Fig. 4). However, γ_2 mRNA is significantly enriched in the medial mammillary nucleus, and γ_3 transcripts are rare in all of these areas. Both γ_1 and γ_2 transcripts are present in substantia nigra (reticulata), again with γ_3 being absent. The γ_2 and γ_3 probes failed to label any white matter tracts (Figs. 2–4). However, an unusual feature of γ_1 autoradiographs is the consistent absence of a signal boundary between (e.g.) cortex and caudate putamen, suggesting the presence of γ_1 mRNA in corpus callosum. Cerebellar white matter tracts, however, remain unlabeled (Fig. 3).

Electrophysiology. Whole-cell recordings from appropriately transfected 293 cells showed that the γ_3 subunit imparts similar properties on GABA-gated $\alpha_1\beta_2\gamma_x$ receptor channels as the γ_2 subunit. Importantly, these include a main channel conductance state (Fig. 5 *A* and *B*) of 30.2 ± 3.5 pS (mean ± SD, n = 10 patches) with few lower conductance openings, similarly to that reported for receptors incorporating the γ_2 subunit and larger than receptors containing only α and β subunits (8, 16). The $\alpha_1\beta_2\gamma_3$ receptor is less sensitive to Zn^{2+} than the $\alpha_1\beta_2$ receptor (Table 1), in agreement with a previous report (23), showing that the presence of a γ subunit impairs $GABA_A$ channel sensitivity to this ion. In addition, the γ_3 subunit like γ_2, when assembled with α and β subunits, forms receptor channels whose activity can be positively and negatively modulated by compounds that interact with the central BZ site (Fig. 5*C* and Table 1). Thus, flunitrazepam potentiates, and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) reduces, $GABA_A$ currents at $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_3$ receptor channels to comparable levels. However, the diazepam and zolpidem-induced potentiations seem less pronounced at $\alpha_1\beta_2\gamma_3$ than at $\alpha_1\beta_2\gamma_2$ receptors. The simplest explanation is that some allosteric compounds have different efficacies at γ_2- and γ_3-subunit-containing receptors. Although differential sensitivity to GABA at these receptors may also play a role it cannot account for these observations, considering the low ratio of test responses to maximal $GABA_A$ currents used by us in all cells analyzed.

Pharmacology. Both $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_3$ receptors show high-affinity binding of BZ site ligands and of the GABA analog [^{3}H]muscimol (Table 2). The rank order of potency for the displacement of [^{3}H]Ro 15-1788 by BZ site ligands was identical for the $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_3$ receptors, but the K_i values differed more than two orders of magnitude. Whereas the estimated ratio of affinities of γ_3 versus γ_2 containing receptors was close to unity for inverse agonists and antagonists of the modulatory BZ site on the $GABA_A$ receptors, this ratio increased considerably for BZ site agonists, particularly for the imidazopyridine zolpidem (25). This data suggests that most agonistic BZ ligands may interact poorly with $GABA_A$ receptor subtypes containing a γ_3 variant in place of γ_2. However, some agonists (e.g., midazolam) may target γ_3-containing receptors, suggesting that these receptors may contribute to the activity profiles of select BZ site compounds.

DISCUSSION

There are now three γ variants that impart distinctive properties to $GABA_A$ receptors assembled from α, β, and γ subunits as judged from *in vitro* studies (5, 7, 10, 26). These properties are the responsiveness to compounds that modulate GABA-gated chloride currents at the BZ site, a large

Table 2. Pharmacology of $\alpha_1\beta_2\gamma_3$ receptors

Ligand	Ligand concentration, nM	% [^{3}H]Ro 15-1788 maximal binding	$\alpha_1\beta_2\gamma_3$ receptor K_i^*, nM	$\alpha_1\beta_2\gamma_2$ receptor K_i, nM	$K_i^*(\gamma_3)/K_i(\gamma_2)$
Zolpidem	30,000	54 ± 1	5500	30	180
2-Oxoquazepam	3,000	58 ± 7	540	20	30
Diazepam†	3,000	51 ± 5	670	16	44
Flunitrazepam†	1,000	55 ± 8	220	2	90
Midazolam	150	52 ± 6	27	2.4	12
β-CCM	30	54 ± 2	5.4	2	3
Ro 15-4513	20	54 ± 5	5.5	6	1

The unlabeled ligands were tested at several concentrations for their ability to displace [^{3}H]Ro 15-1788. At the concentration listed, these compounds displaced [^{3}H]Ro 15-1788 to ≈50% in three experiments. The tracer was at 4.5-fold above its K_d value experimentally determined to be 2.0 ± 0.2 nM in three experiments. The inhibitory values were used to predict rough K_i (K_i^*) values (24). β-CCM, methyl β-carboline-3-carboxylate.
†Full competition curves were determined for diazepam and flunitrazepam and used to derive K_i values for these compounds at $\alpha_1\beta_2\gamma_3$ receptors. These K_i values were 710 ± 120 nM for diazepam and 180 ± 50 nM for flunitrazepam, in good agreement with the K_i^* values predicted from single concentration points. The ratios of K_i^* values at $\alpha_1\beta_2\gamma_3$ receptors vs. K_i values at $\alpha_1\beta_2\gamma_2$ receptors are from refs. 4–6. The ratio of K_d values of [^{3}H]Ro 15-1788 at $\alpha_1\beta_2\gamma_3$ vs. $\alpha_1\beta_2\gamma_2$ receptors is roughly 4.

single-channel conductance of ≈30 pS, and a reduced rate of desensitization relative to receptors assembled from α and β subunits (8). In addition, γ variants appear to relieve a noncompetitive Zn^{2+} block (23).

As with α variants (1, 2, 4–6, 12), γ variants differ in the extent to which they modify the functional properties of $GABA_A$ receptors, particularly the affinity of compounds interacting with the BZ site on $\alpha_1\beta_2\gamma_x$ receptors. Whereas agonists bind with high affinity to $\alpha_1\beta_2\gamma_2$ (5), they exhibit affinities reduced by approximately two orders of magnitude at $\alpha_1\beta_2\gamma_3$ receptors. However, antagonists and inverse agonists display similar affinities at $\alpha_1\beta\gamma_2$ and $\alpha_1\beta\gamma_3$ receptors. Electrophysiological studies revealed that the efficacy of agonists at the BZ recognition site of $GABA_A$ receptors is dependent upon the molecular variant of the α subunit. However, receptors differing in their γ subunit present disparate pharmacological profiles: γ_2-subunit expression confers high sensitivity to BZ site agonist modulation of GABA responses (7, 26) whereas the γ_1 subunit (26) and γ_3 subunit (present results) characterize receptors with different responses to modulation by many ligands acting at the BZ site. Interestingly, $GABA_A$ receptors containing the γ_3 subunit are sensitive to flunitrazepam and DMCM, as are γ_2-containing receptors, but lack the high-efficacy response to diazepam and zolpidem.

Based on the pronounced anatomical restriction of γ_3 mRNA, it would seem that this subunit contributes to a population of specialized $GABA_A$ receptors (e.g., in the medial geniculate thalamic nucleus, an area involved in auditory processing). Although we have used the $\alpha_1\beta_2$ combination as a reference standard for the systematic comparison of the properties of the γ subunits, the γ_3 subunit may occur with other subunit partners *in vivo*. For example, in the medial geniculate thalamic nucleus, the only subunit mRNAs present at significant levels are α_1, α_4, β_2, δ, and γ_3 (27). Thus, the $\alpha_1\beta_2\gamma_3$ combination is a subset of these receptor constituents and may indeed be the genuine *in vivo* combination in this nucleus.

Note. As this report was being prepared for publication, the sequence of a mouse γ_3 subunit was reported (28). No functional data were demonstrated for the mouse γ_3 homologue. From a brief *in situ* hybridization analysis, signals of very low and uniform intensity were observed throughout the mouse brain, with hippocampus being the only structure strongly demarcated. It was concluded that the distribution of γ_3 mRNA largely resembled that of γ_2 transcripts (28). The γ_3 oligonucleotide probe used in the mouse brain study overlapped one of our probes by 34 bases. Hence, the γ_3 mRNA distribution in rat brain would appear to be different from that reported in mouse and may possibly arise from a species difference.

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