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γ-Aminobutyric Acid Type A Receptors in the Rat Brain Can Contain Both γ2 and γ3 Subunits, but γ1 Does not Exist in Combination with Another γ Subunit

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SUMMARY

Antibodies specific for the γ1, γ2, and γ3 subunits of the γ-aminobutyric acid $(GABA)_A$ receptor have been used to probe the composition of naturally occurring $GABA_A$ receptors in the rat brain. Most $GABA_A$ receptors contain at least one of these three subunits. The percentage of each, determined by immunoprecipitation of [^{3}H]muscimol binding, was 11 ± 1%, 59 ± 3%, and 14 ± 2% for γ1, γ2, and γ3 subunits, respectively. Receptors containing γ2 or γ3 subunits were labeled by benzodiazepine site ligands with high affinity, whereas γ1-containing receptors could be labeled only by [^{3}H]muscimol. Receptors immunoprecipitated by anti-γ2 or anti-γ3 antibodies were labeled with [^{3}H]Ro 15-1788 with similar affinities (K_d for anti-γ2-immunoprecipitated receptors, 1.9 nM; K_d for anti-γ3-immunoprecipitated receptors, 1.7 nM). Immunoprecipitation or Western blot analysis of $GABA_A$ receptors solubilized from rat cerebellar or whole-brain preparations indicated that γ1 was not present coassembled with any other γ subunit. Western blot analysis of receptors purified on α-specific immunoaffinity resins showed that γ1 was predominantly assembled with the α2 subunit. Some $GABA_A$ receptors may contain more than one type of γ subunit. Quantitative immunoprecipitation and Western blot analysis both indicated that γ2 and γ3 subunits can exist in the same receptor complex. A large proportion of $GABA_A$ receptors immunopurified on a γ3 affinity resin also appeared to contain a γ2 subunit. In contrast, when receptors were purified on a γ2 affinity resin a small proportion also appeared to contain a γ3 subunit. We conclude that most γ1-containing receptors have no other γ subunit in the same receptor complex but some $GABA_A$ receptors contain both γ2 and γ3 subunits.

Neuronal inhibition in the brain is largely mediated through GABA-gated chloride ion channels collectively known as $GABA_A$ receptors. In the mammalian brain a large family of $GABA_A$ receptors exist, the structure of which is defined by the coassembly of probably five subunits, selected from a repertoire of at least 14 (α1–α6, β1–β3, γ1–γ3, and δ) (for review, see Refs. 1–4). In addition to a GABA binding site, these proteins have modulatory sites for barbiturates, steroids, and benzodiazepines (for review, see Refs. 5 and 6). The combination of subunits present dictates the pharmacological properties of the benzodiazepine site, with contributions from both α (7–11) and γ (12–15) subunits. Benzodiazepine binding sites present on $GABA_A$ receptors have been grouped into two types. Those with high affinity for CL 218872 (K_i of ~100 nM) and for other compounds including zolpidem and 2-oxoquazepam have been designated as benzodiazepine type 1 receptors. Those with lower affinity for CL 218872 (K_i of ~1 μM) and other compounds have been designated as benzodiazepine type 2 receptors. The type of the α subunit present correlates with pharmacologically defined subtypes of $GABA_A$ receptors. When transfected into cells with a γ2 and β subunit, the α1 subunit confers a benzodiazepine type 1 pharmacology (7), the α2, α3 (7), and α5 (8) subunits confer a benzodiazepine type 2 pharmacology, and a third class of $GABA_A$ sites with low affinity for many benzodiazepines is created by receptors that contain either α4 or α6 subunits (9, 11). The presence of a γ subunit appears to be necessary for benzodiazepine modulation in transfected cells, although the pharmacological profiles produced by γ1, γ2, or γ3 subunits are clearly different (11–16). Little information is available regarding how α and γ subunits are paired in naturally occurring receptors and whether more than one of these subunits is present in a single receptor complex.

We have previously used polyclonal antibodies, raised against the bacterially expressed putative cytoplasmic loop regions of α subunits, to immunologically separate naturally occurring populations of $GABA_A$ receptors, and we have investigated the ligand-binding properties and subunit composition of the receptors. We, and others, have concluded from such experiments that most receptors contain only one type of α subunit, but a

ABBREVIATIONS: GABA, γ-aminobutyric acid; ECL, enhanced chemiluminescence; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

small proportion may contain two different types (17–21). We have adopted similar approaches to investigations of the γ subunits and we report here that receptors that contain two γ subunits are also present in rat brain.

Materials and Methods

[*N-methyl*-^{3}H]Flunitrazepam (81.8 Ci/mmol), Protein A-horseradish peroxidase, and the ECL detection kit were from Amersham International; [*methylene*-^{3}H]muscimol (17.1 Ci/mmol), [*N-methyl*-^{3}H]Ro 15–1788 (79.8 Ci/mmol), and [7,9-^{3}H]Ro 15–4513 (29 Ci/mmol) were from New England Nuclear DuPont (Hertfordshire, UK). Peroxidase-conjugated IgG raised to rabbit IgG (P217) was from DAKO (Denmark). TMBlue precipitating reagent was from Universal Biologicals (London, UK). Ro 15–4513, Protein A-Sepharose, deoxycholate, flunitrazepam, and all other reagents were from Sigma (UK).

Generation of anti-γ1, anti-γ2, and anti-γ3 antisera. Expression of the unique parts of the putative cytoplasmic loop proteins from the γ1, γ2, and γ3 subunits was performed in a manner similar to that used for the other $GABA_A$ receptor subunits, as described elsewhere (17, 22). All three proteins were expressed as fusion proteins. The amino-terminal 24 amino acids were derived from the first 11 amino acids of gene 10 (the major capsid protein of T7 bacteriophage) followed by Arg-Gly-Ser-Glu-Leu-Glu-Ile-Cys-Ser-Trp-Tyr-His-Gly, derived from the polylinker sequence of pRSET5a. The carboxyl-terminal portions were contributed by His^{320}–Tyr^{369}, Leu^{317}–Tyr^{367}, and Asn^{321}–Val^{388} of γ1 (13), γ2L (23), and γ3 (14), respectively. Briefly, the regions encoding cytoplasmic loop polypeptides were amplified from rat whole-brain cDNA (1 μg) for γ1 and γ3 or from a cDNA clone of bovine γ2L (23), incorporating an *Eco*RI site in the upstream primer and a *Hind*III site after a stop codon in the downstream primer. Polymerase chain reaction products were subcloned into pRSET5a and the recombinant proteins were expressed in *Escherichia coli* using methods described previously (17, 22). Antibodies were also raised against a fourth expression protein. This polypeptide was not expressed as a fusion protein but contained only a longer region of the γ2L subunit (Leu^{317}–Met^{403}), as described by Whiting *et al.* (24). Both γ2 antigens contained the eight extra amino acids present in γ2L (Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys), which occur between Pro^{337} and Ala^{338} (19). The bacterially expressed fusion proteins were purified by preparative SDS-PAGE, and rabbits were immunized subcutaneously with 100-μg aliquots emulsified with Freund's complete adjuvant (1/1) and were boosted at monthly intervals with 50 μg of polypeptide emulsified with Freund's incomplete adjuvant. Blood samples were taken from rabbits 7 days after each boost and the presence of antibodies was assayed by the ability of the antiserum to immunoprecipitate specific [^{3}H]muscimol binding sites from a deoxycholate-solubilized membrane preparation from rat whole brain.

Antibodies raised against the intracellular loop polypeptide γ2(Leu^{317}–Met^{403}) could potentially cross-react with both γ1 and γ3 subunits, because the polypeptide contained stretches of 17 and nine amino acids, respectively, common to these subunits. A peptide from the γ1 subunit (CASFFCCFEDCRTGAWRHGRIHIRI) that spans the common region was synthesized to test more directly whether the antibody raised against γ2L(Leu^{317}–Met^{403}) was specific. The antiserum reacted very weakly with the common peptide on a Western blot and was unable to detect <10 μg of the synthetic peptide at a dilution of 1/500. Initial studies with the common polypeptide therefore indicated that the anti-γ2L(Leu^{317}–Met^{403}) antiserum was, at best, weakly cross-reactive with γ1 and/or γ3. It could not be regarded as specific for the γ2 subunit until additional experiments exploring its ability to detect expressed polypeptides or to immunoprecipitate receptors of defined composition had been carried out.

Immunoprecipitation and ligand binding. Immunoprecipitation of $GABA_A$ receptors solubilized from rat brain was carried out using antibodies bound to Protein A-Sepharose, as described previously (17, 22). For quantitative immunoprecipitation experiments the amount of antiserum required for maximal immunoprecipitation was established by first incubating varying amounts of antiserum (1–60 μl) with 30 μl of Protein A-Sepharose beads, in a total volume of 1 ml, for 1 hr at room temperature. The Protein A-antibody conjugates were washed three times with Tris-buffered saline and then incubated at 4° overnight with 0.4 ml of deoxycholate-solubilized rat brain membrane preparation. Binding of [^{3}H]Ro 15–1788, [^{3}H]Ro 15–4513, and [^{3}H]muscimol was carried out with both beads and supernatant. For saturation analysis of immunoprecipitated receptors containing the various γ subunits, 150 μl of antiserum were incubated with 600 μl of Protein A-Sepharose, followed by overnight incubation with 10 ml of solubilized membranes from whole rat brain (17). Under these conditions 0.5, 3.1, and 0.8 pmol of [^{3}H]muscimol sites were immunoprecipitated by anti-γ1, anti-γ2, and anti-γ3 antibodies, respectively.

Radioligand binding studies were carried out in 10 mM Tris·HCl, 1 mM EDTA, pH 7.4, in a total volume of 0.5 ml, for 1 hr at room temperature before termination by filtration through Whatman GF/C filters, followed by washing three times with 3 ml of 5 mM Tris·HCl, pH 7.4, and scintillation counting. Nonspecific binding was determined using 100 μM GABA for [^{3}H]muscimol binding and 10 μM Ro 15–4513 for both [^{3}H]Ro 15–4513 and [^{3}H]Ro 15–1788.

Immunoaffinity purification and Western blot analysis. Immunoaffinity purification of the γ-containing populations was achieved using an IgG-Sepharose affinity column. IgG was purified from 2 ml of the respective antiserum by using a Protein A-Sepharose affinity resin and was coupled to CNBr-activated Sepharose 4B (1 ml) using methods described previously (17). Receptors purified with the IgG affinity resins were subjected to 12% SDS-PAGE, transferred to nitrocellulose, and probed with a 1/500 dilution of primary antibody, unless otherwise stated, using the protocol detailed previously (17). Detection of the antibody was achieved by incubating the blot with horseradish peroxidase-linked Protein A, followed by visualization with TMBlue or the ECL detection kit as indicated, according to the manufacturer's instructions.

Results

Expression of putative cytoplasmic loop polypeptides and characterization of antisera. The bacterially expressed γ1, γ2, and γ3 putative cytoplasmic loop polypeptides had molecular masses on 15% SDS-PAGE of 10,500, 13,800, and 14,100 Da, respectively. The molecular masses calculated from their known amino acid compositions were 9,192, 11,117, and 12,257 Da for γ1, γ2, and γ3, respectively. The longer γ2 polypeptides γ2L(Leu^{317}–Met^{403}) and γ2S(Leu^{317}–Met^{403}) had molecular sizes of 15,500 and 14,500 Da, respectively, in reasonable accord with their predicted molecular masses of 12,850 and 11,980 Da (24). All of the proteins were expressed to high levels, as shown in Fig. 1, facilitating their purification in sufficient quantity to generate polyclonal antisera. Antisera raised against each protein [γ1, γ2(Leu^{317}–Tyr^{367}), γ3, and γ2(Leu^{317}–Met^{403})] were tested for specificity by their ability to detect all of the proteins [γ1, γ2, γ3, γ2L(Leu^{317}–Met^{403}), and γ2S(Leu^{317}–Met^{403})] in crude bacterial lysates. The sequences of γ1(His^{320}–Tyr^{369}), γ2(Leu^{317}–Tyr^{367}), and γ3(Asn^{321}–Val^{388}) were substantially different from each other, with <30% overall identity and with a maximum of three consecutive identical amino acids. No cross-reactivity of these antibodies would therefore be expected. In Fig. 2 it can be seen that the γ1 antiserum is able to detect only the γ1 recombinant polypeptide. γ2(Leu^{317}–Tyr^{367}), γ2L(Leu^{317}–Met^{403}), and γ2S(Leu^{317}–Met^{403}) recombinant polypeptides could all be detected by antisera raised against both γ2(Leu^{317}–Tyr^{367}) and γ2(Leu^{317}–Met^{403}). The expressed γ3 recombinant protein was detected only by the anti-γ3 antiserum, confirming the specificity of all

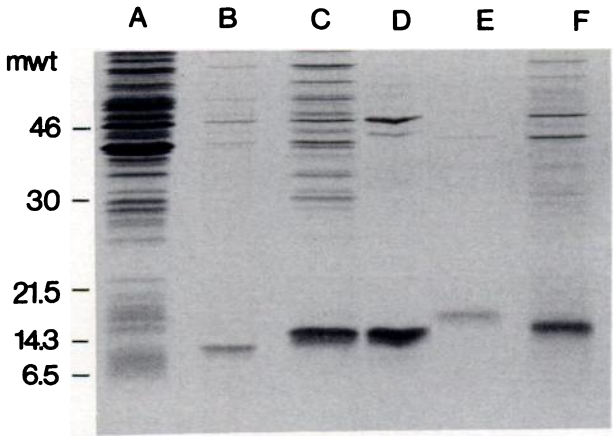

Fig. 1. Expression of putative cytoplasmic loop proteins in *E. coli*. BL21DE3(LysS) *E. coli* were transformed with plasmid containing either no insert (*lane A*), γ1(His^{320}–Tyr^{369}) (*lane B*), γ2(Leu^{317}–Tyr^{367}) (*lane C*), γ3(Asn^{321}–Val^{368}) (*lane D*), γ2L(Leu^{317}–Met^{403}) (*lane E*), or γ2S(Leu^{317}–Met^{403}) (*lane F*). Cells were induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) for 3 hr, and crude lysates, prepared as described in Materials and Methods, were subjected to 15% SDS-PAGE and stained with Coomassie blue. Molecular size markers (× 1000) are shown on the *left*.

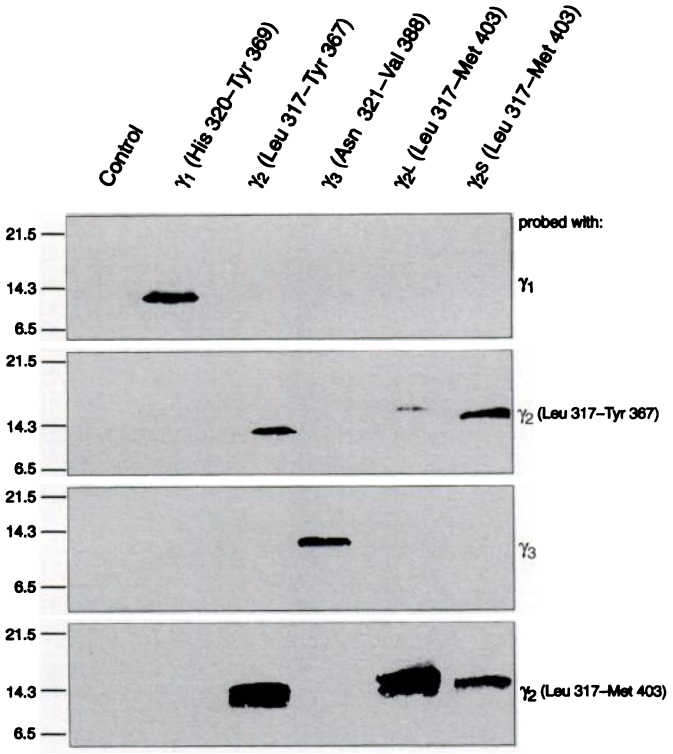

Fig. 2. Cross-reactivity of anti-γ antibodies. Cross-reactivity of the various anti-γ antibodies was investigated by Western blot analysis. Approximately 1 mg of each purified recombinant polypeptide was subjected to 15% SDS-PAGE, as indicated, and transferred to nitrocellulose. The layout for each blot was the same and the control was lysates from *E. coli* transformed with vector alone, containing no insert, and induced with isopropyl-β-D-thiogalactopyranoside. Western blots were probed with antiserum (1/500) raised against γ1(His^{320}–Tyr^{369}), γ2(Leu^{317}–Tyr^{367}), γ3(Asn^{321}–Val^{368}), or γ2(Leu^{317}–Met^{403}) as indicated.

of the antisera. Antisera raised against the longer γ2 polypeptide, γ2(Leu^{317}–Met^{403}), did not detect either γ1 or γ3 polypeptides by Western blot (Fig. 2), despite the presence of common sequences including a 17-amino acid stretch common to γ1 and a stretch of nine amino acids also found in γ3. For a given dilution, anti-γ2(Leu^{317}-Met^{403}) antiserum detected all of the expressed γ2 polypeptides more strongly by Western blot than did antiserum raised against γ2(Leu^{317}–Tyr^{367}), and for this reason the former was used in further studies. None of the antisera used in this study was able to detect its respective subunit in Western blot analysis of crude membranes prepared from rat brain. This is consistent with other studies using antisera raised against different regions of the γ2 subunits (25, 26). Despite its abundance in brain, relative to other GABA$_A$ subunits such as, for example, the α1 subunit, the γ2 subunit has been visualized only after purification of the receptor by either ligand affinity or immunoaffinity purification (25–28). The γ2 subunit is difficult to detect by Western blot (29), and as a class the γ subunits may therefore be particularly sensitive to proteolytic degradation.

Immunoprecipitation of receptors from cells expressing α1β3γ2 or α1β3γ3 recombinant human GABA$_A$ receptors. Although antisera showed no detectable cross-reactivity when tested against expressed polypeptides on a Western blot, it was also important to demonstrate that they showed no cross-reactivity between the subunits when they are present in their native conformation in intact GABA$_A$ receptors. This was tested by immunoprecipitating receptor of defined composition from human embryonic kidney 293 cells transiently transfected with either α1β3γ2 or α1β3γ3 human subunit combinations (30). The anti-γ2(Leu^{317}–Met^{403}) antiserum, which gave the most intense signal on a Western blot, was compared with the anti-γ3 antiserum. Receptors, solubilized from cell membranes with deoxycholate (0.7%), were incubated for 14 hr at 4° with Protein A-Sepharose coupled to either anti-γ2 or -γ3 antiserum, and immunoprecipitated receptors were assayed with [^{3}H]muscimol, as described in Materials and Methods. Receptors from cells transfected with α1β3γ2 subunits could be immunoprecipitated only with anti-γ2 antiserum. Solubilized receptor preparations contained 236 fmol of [^{3}H]muscimol binding sites/ml; control irrelevent hyperimmune rabbit antiserum immunoprecipitated 5.5 fmol (2.3%), anti-γ2(Leu^{317}–Met^{403}) antiserum immunoprecipitated 231 fmol (98%), and anti-γ3 antiserum immunoprecipitated 1.8 fmol (0.7%) (one experiment). Similarly, receptors from cells transfected with α1β3γ3 subunits could be immunoprecipitated with anti-γ3 antiserum only. Solubilized receptor preparations contained 91 fmol of [^{3}H]muscimol binding sites/ml; control irrelevent antiserum immunoprecipitated 4.9 fmol (5.3%), anti-γ2 antiserum immunoprecipitated 5.7 fmol (6.2%), and anti-γ3 antiserum immunoprecipitated 83 fmol (92%) (one experiment). Therefore, these data, together with the data from Fig. 2 and Tables 1 and 2, show that the anti-γ2(Leu^{317}–Met^{403}) and -γ3 antisera

TABLE 1

Saturation analysis of [^{3}H]muscimol and [^{3}H]Ro 15-1799 binding to GABA$_A$ receptors immunoprecipitated with anti-γ1, anti-γ2(Leu^{317}–Met^{403}), or anti-γ3 antiserum

Data shown are the mean ± standard error of three or four paired experiments.

	K_d		B_{max} [^{3}H]muscimol/ B_{max} [^{3}H]Ro 15-1788
	[^{3}H]Muscimol	[^{3}H]Ro 15-1788	
	nM		
γ1	5.3 ± 1.2		
γ2	5.9 ± 0.8	1.9 ± 0.6	2.4 ± 0.2
γ3	7.2 ± 1.2	1.7 ± 0.3	2.3 ± 0.4

TABLE 2

Immunoprecipitation of $GABA_A$ receptor binding sites by anti-γ1(His^{320}–Tyr^{369}), anti-γ2(Leu^{317}–Tyr^{367}), and anti-γ3(Asn^{321}–Val^{388})

The percentage immunoprecipitation was calculated for each experiment by taking the amount of receptor in the solubilized material as 100%. For [^{3}H]muscimol binding, receptors were gel-filtered through Sephadex G-25, to remove endogenous GABA, before radioligand binding. The "observed" value is the percentage of receptor immunoprecipitated after inclusion of both antibodies together. The "theoretical" value is that obtained by summing the percentages of receptor immunoprecipitated by the antibodies individually. Data are the mean ± standard error of three to five determinations.

Antibody	[^{3}H]Muscimol binding		[^{3}H]Ro 15-1788 binding	
	Observed	Theoretical	Observed	Theoretical
	%		%	
A. Cerebellum				
γ1	9.3 ± 1.6		0	
γ2	68 ± 1		108 ± 5	
γ1 ± γ2	89 ± 6	77	109 ± 6	
B. Whole brain				
γ1	11 ± 1		0	
γ2	59 ± 3		78 ± 6	
γ3	14 ± 2		18 ± 3	
γ1 + γ2	83 ± 4	70	77 ± 4	78
γ2 + γ3	66 ± 7	73	96 ± 7	96
γ1 + γ3	24 ± 6	25	11 ± 4	18

are, as far as could be tested, specific for their respective subunits.

Immunoprecipitation of $GABA_A$ receptors from rat brain. Antisera raised against the four recombinant proteins were able to immunoprecipitate $GABA_A$ receptors from rat brain to differing extents. The ability of anti-γ antisera to immunoprecipitate both [^{3}H]muscimol and [^{3}H]Ro 15–1788 binding sites was compared (Fig. 3), and the percentages of receptor immunoprecipitated from whole brain or cerebellum are summarized in Table 2.

[^{3}H]Muscimol labels $GABA_A$ receptors at the GABA binding site. It is assumed that [^{3}H]muscimol, being a structural analogue of the endogenous ligand GABA, labels the entire $GABA_A$ receptor population. Should GABA receptors exist that do not bind [^{3}H]muscimol with high affinity, they would not be included in our analyses. Assuming that all receptors are labeled and under conditions where receptor, not Protein A or antibody, is limiting, the proportion of [^{3}H]muscimol binding sites immunoprecipitated by each of the antibodies should reflect the abundance of the receptor subtypes in brain. Experiments were carried out with 30 μl [γ1, γ2(Leu^{317}–Met^{403}), and γ3] or 60 μl [γ2(Leu^{317}–Tyr^{367})] of antiserum, immobilized on 60 μl of Protein A-Sepharose beads, and 0.4 ml of solubilized rat brain preparation, which routinely contained 0.3–0.5 pmol of receptor. Under these conditions the amount of antibody was saturating (see Fig. 3) and was assumed to immunoprecipitate all available sites. Of the three γ subunits the least abundant was γ1, which precipitated only 11 ± 1% (four experiments) of [^{3}H] muscimol binding sites, γ3 immunoprecipitated 14 ± 2% (six experiments) of [^{3}H]muscimol sites, and γ2 was the most abundant, immunoprecipitating 59 ± 3% (five experiments) of all $GABA_A$ receptors. The anti-γ2(Leu^{317}–Met^{403}) antiserum immunoprecipitated 62 ± 4% (four experiments) of $GABA_A$ receptors, a value not significantly different from that for γ2(Leu^{317}–Tyr^{367}), confirming its specificity for this subunit. This is the first demonstration of the relative abundance of γ1, γ2, and γ3 subunit proteins expressed in rat brain and is consistent with the observed relative abundance of mRNA for

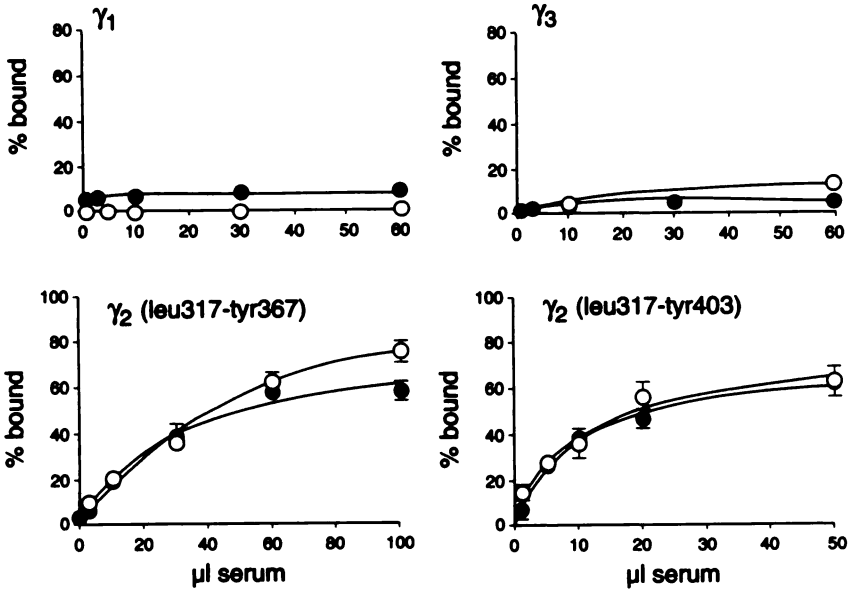

Fig. 3. Immunoprecipitation of [^{3}H]muscimol (●) and [^{3}H]Ro 15–1788 (○) binding sites by antibodies against γ1, γ2(Leu^{317}–Tyr^{367}), γ3, and γ2(Leu^{317}–Met^{403}). Protein A-Sepharose was incubated with various volumes of antiserum (as indicated) for 1 hr, washed extensively, and incubated with $GABA_A$ receptors solubilized from rat whole brain (0.4 ml). Precipitated receptor was washed three times with Tris-buffered saline/0.1% Tween (1 ml), and binding of [^{3}H]muscimol and [^{3}H]Ro 15–1788 to the receptor-antibody-Protein A-Sepharose complex was then performed as described in Materials and Methods.

these subunits (31, 32). Antibodies to γ1 and γ3 have not been described previously. Data presented here for immunoprecipitation of receptors containing the γ2 subunit are consistent with the work of Duggan *et al.* (25), who found that 37–69% of [^{3}H]muscimol sites could be immunoprecipitated by γ2 antipeptide antibodies from various regions of bovine brain, and with the work of Khan *et al.* (26), who reported immunoprecipitation of 70% of [^{3}H]muscimol binding sites from rat cerebral cortex with an antibody raised against the intracellular loop of the γ2 subunit.

When GABA$_A$ receptors were labeled at the benzodiazepine site with [^{3}H]Ro 15–1788 a different picture emerged. The γ1 antiserum was unable to immunoprecipitate significant numbers of [^{3}H]Ro 15–1788 binding sites (Fig. 3). Antibodies to γ2 and γ3 immunoprecipitated 76 ± 4% and 21 ± 3% (four experiments), respectively, of the total solubilized benzodiazepine binding sites. The anti-γ2(Leu^{317}–Met^{403}) antibodies immunoprecipitated 80 ± 4% of the [^{3}H] Ro 15–1788 binding sites, a value not significantly different from that for γ2(Leu^{317}–Tyr^{367}). Again, this is consistent with the work of Khan *et al.* (26), who reported a large proportion (87%) of [^{3}H]flunitrazepam binding sites immunoprecipitated from rat cortex by anti-γ2 antisera, and Duggan *et al.* (25), who reported that 68–83% of sites were immunoprecipitated from various regions of bovine brain. The inability of [^{3}H]Ro 15–1788 to label GABA$_A$ receptors immunoprecipitated with the anti-γ1 antiserum also extended to other ligands for the benzodiazepine site. [^{3}H]Flunitrazepam, [^{3}H]Ro 15–4513, [^{3}H]CGS-8216, and [^{3}H]zolpidem were also ineffective as ligands for γ1-containing receptors (data not shown). These ligands were all able to label receptors immunoprecipitated with anti-γ2(Leu^{317}–Tyr^{367}), anti-γ3, or anti-γ2(Leu^{317}–Met^{403}) antibodies (data not shown). When all [^{3}H]muscimol binding sites had been immunoprecipitated by a combination of antisera, no further benzodiazepine binding sites remained in solution, indicating that populations of receptors that have a benzodiazepine binding site are contained within those that have a high affinity [^{3}H]muscimol binding site.

To investigate further the pharmacology of receptors containing each γ subunit, larger amounts of receptor were immunoprecipitated as described in Materials and Methods. Scatchard analysis was performed on receptors immunoprecipitated with anti-γ1, -γ2(Leu^{317}–Tyr^{367}), and -γ3 antisera. Both [^{3}H]muscimol and [^{3}H]Ro 15–1788 (for γ2 and γ3) were used as ligands, as summarized in Table 1. Immunoprecipitation had no significant effect on the affinity of radioligand for the GABA$_A$ receptor. ([^{3}H]Muscimol labels solubilized receptors that have been gel-filtered, to remove endogenous GABA, with a K_d in the range of 5–10 nM and [^{3}H]Ro 15–1788 labels receptors in membranes or solubilized preparations with a K_d in the range of 1–3 nM.) There was no difference in the affinity of [^{3}H]Ro 15–1788 for γ2- or γ3-containing receptors (Fig. 4; Table 1). Similarly, there was no significant difference between the affinities of [^{3}H]muscimol for receptors containing either γ1, γ2, or γ3 subunits (Fig. 5; Table 1). For anti-γ2- and anti-γ3-immunoprecipitated receptors the observed ratio of [^{3}H]muscimol to [^{3}H]Ro 15–1788 binding sites of 2.4:1 would, within experimental limits, suggest that there are two GABA$_A$ binding sites and one benzodiazepine binding site on each receptor molecule. Although such experiments allow a comparison of the number of sites for each ligand on an individual receptor

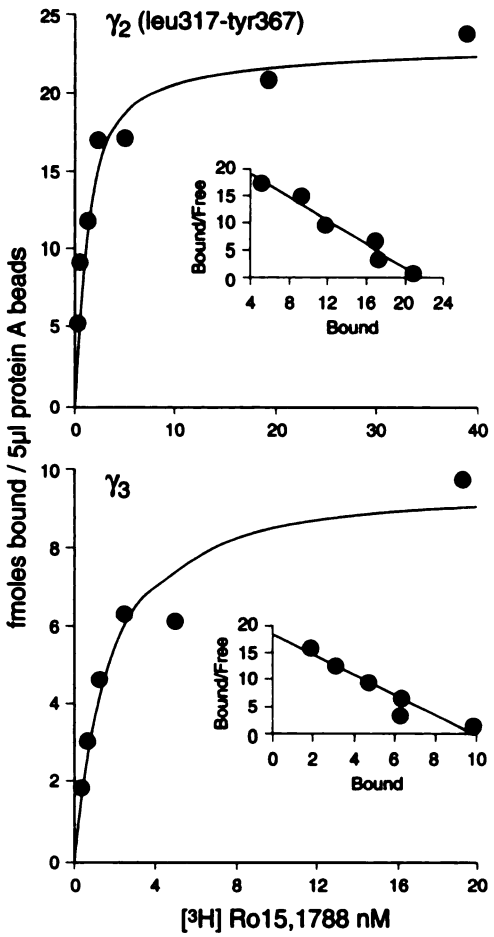

Fig. 4. Saturation curves and Scatchard analysis of [^{3}H]Ro 15–1788 binding to immunoprecipitated GABA$_A$ receptors. GABA$_A$ receptors from 10 ml of solubilized rat brain membranes were immunoprecipitated with 150 µl of anti-γ2(Leu^{317}–Tyr^{367}) or anti-γ3 antiserum bound to 600 µl of Protein A-Sepharose. Saturation analysis and Scatchard plots (*insets*) [(where the *x*-axis is bound (fmol/5 µl of Protein A-Sepharose beads) and the *y*-axis is bound/free (fmol/5 µl of Protein A-Sepharose beads/nM)] were best fitted using ENZfit. Data shown are from one experiment (γ2, B_{max} = 22.7 fmol/5 µl of Protein A-Sepharose beads, K_d = 1.04 nM; γ3, B_{max} = 9.75 fmol/5 µl of Protein A-Sepharose beads, K_d = 1.47 nM) and are representative of four similar experiments.

subtype, they do not allow a comparison of B_{max} values for each subtype, because the experiments are carried out under conditions where the antibody is limiting and saturated with receptor. Further analysis of the pharmacology of the benzodiazepine site on γ2- and γ3-containing receptors was not possible because competition curves had low Hill slopes whose components could not easily be resolved. The n_H values for anti-γ2-immunoprecipitated receptors were as follows: zolpidem, 0.65 ± 0.02; CL 218872, 0.62 ± 0.03; flunitrazepam, 0.85 ± 0.03; Ro 15–1788, 0.84 ± 0.14. The n_H values for anti-γ3-immunoprecipitated receptors were as follows: zolpidem, 0.53 ± 0.01; CL 218872, 0.24 ± 0.03; flunitrazepam, 0.67 ± 0.05; Ro 15–1788, 0.91 ± 0.33. The n_H values for solubilized receptor preparations were as follows: zolpidem, 0.55 ± 0.10; CL 218872, 0.58 ± 0.02; flunitrazepam, 0.95 ± 0.03; Ro 15–1788, 0.91 ± 0.04. The low Hill slopes presumably were derived from the pairing of γ subunits with multiple α subunits and were observed for both γ2- and γ3-containing receptors.

Is there more than one type of γ subunit in a receptor

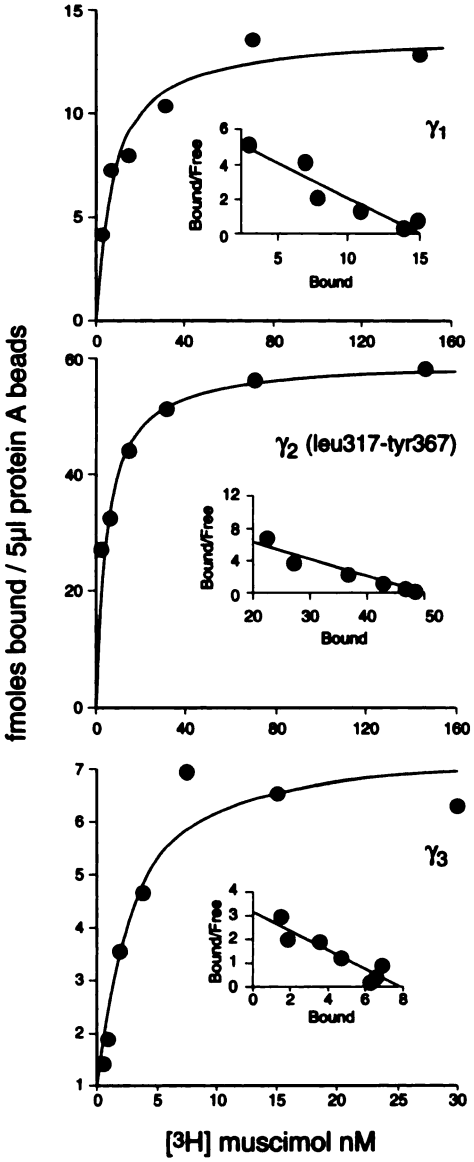

Fig. 5. Saturation curves and Scatchard analysis of [^{3}H]muscimol binding to immunoprecipitated $GABA_A$ receptors. $GABA_A$ receptors solubilized from whole-brain membranes were immunoprecipitated with anti-γ1, anti-γ2(Leu^{317}–Tyr^{367}), or anti-γ3 antibodies as indicated. Saturation analysis and Scatchard plots (*insets*) were best fit using ENZfit. Data shown are from one experiment (γ1, B_{max} = 13.6 fmol/5 µl of Protein A-Sepharose beads, K_d = 6.6 nM; γ2, B_{max} = 58.7 fmol/5 µl of Protein A-Sepharose beads, K_d = 5 nM; γ3, B_{max} = 8.2 fmol/5 µl of Protein A-Sepharose beads, K_d = 4.5 nM) and are representative of three similar experiments.

molecule?. Two experimental approaches were taken to resolve whether receptors exist that contain more than one type of γ subunit. Firstly, receptors were immunopurified on γ-specific affinity resins and subjected to Western blot analysis. Secondly, quantitative immunoprecipitation experiments were carried out using combinations of γ subunit-specific antisera.

The IgG fractions purified from anti-γ1, anti-γ2(Leu^{317}–Met^{403}), or anti-γ3 antisera were coupled to CNBr-Sepharose and used to immunopurify $GABA_A$ receptors solubilized from whole rat brain. The γ subunit-specific receptor populations were eluted from the immunoaffinity columns, resolved by SDS-PAGE, Western blotted, and probed with anti-γ1, anti-γ2(Leu^{317}–Met^{403}), and anti-γ3 antisera (Fig. 6). Western blot analysis confirmed that the γ1 subunit does not coexist in a receptor with either the γ2 or γ3 subunit (Fig. 6). However, receptors do exist that contain both γ2 and γ3 subunits (Fig. 6). Although Western blots are not quantitative, it is possible to make some general comments about the proportion of receptors that might contain both a γ2 subunit and a γ3 subunit. Similar amounts of receptor were loaded on the Western blot probed with anti-γ2 antiserum and a strong signal was observed in the lane loaded with receptor purified on a γ3 immunoaffinity resin, comparable to that obtained with receptor immunopurified using a γ2 immunoaffinity resin. This suggests that a large proportion of γ3-containing receptors also contain a γ2 subunit. Conversely, in the Western blot probed with anti-γ3 antiserum, 7 times more anti-γ2-immunopurified receptor was loaded, compared with γ3, in an effort to allow a potentially small population of receptors containing both γ2 and γ3 subunits to be detected. The approximately equal intensities of the bands observed indicated that only a small proportion of γ2-containing receptors also contain a γ3 subunit.

In the quantitative studies receptor was immunoprecipitated from two brain regions, i.e., from cerebellum, where the cells do not appear to express the γ3 subunit (14, 15, 31) (thereby simplifying the analysis), and also from whole brain, to ensure that all receptor populations were included. Immunoprecipitated material was assayed with both [^{3}H]muscimol and [^{3}H]Ro 15–1788. In the cerebellum, when [^{3}H]muscimol was used as a ligand the sum of the percentages of receptors immunoprecipitated by γ1 and γ2 separately was not more than those immunoprecipitated by the two antisera in combination (Table 2), suggesting that these two subunits reside in separate receptor molecules. When [^{3}H]Ro 15–1788 was used as a ligand, all sites in the cerebellum were immunoprecipitated by the anti-γ2 antiserum. In whole brain, 78% of [^{3}H]Ro 15–1788 binding sites were immunoprecipitated by the anti-γ2 antiserum, and this was not increased by inclusion of anti-γ1 antiserum. Therefore, consistent with the Western blot experiments, γ1 and γ2 subunits do not appear to coexist in the same receptor molecule. Immunoprecipitation with a combination of anti-γ1 and anti-γ3 antisera could be tested only in whole brain. The percentage of [^{3}H]muscimol binding sites immunoprecipitated by anti-γ1 and anti-γ3 antisera in combination (24%) (Table 2B) was not

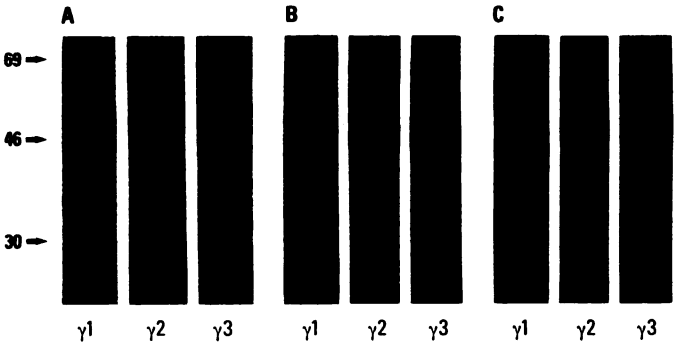

Fig. 6. Western blot analysis of receptors immunopurified on γ1, γ2, and γ3 immunoaffinity resins. The γ1, γ2, and γ3 lanes were loaded with 2, 3.5, and 0.5 pmol of [^{3}H]muscimol binding sites, respectively. After separation by SDS-PAGE and transfer to nitrocellulose, blots were probed with antiserum raised against γ1 (A), γ2(Leu^{317}–Met^{403}) (B), or γ3 (C), at a dilution of 1/500. Bands were visualized using ECL, according to the manufacturer's instructions. Molecular size markers (× 1000) are shown on the *left*.

significantly different from the sum of the sites immunoprecipitated by anti-γ1 and anti-γ3 antisera individually (25%) (Table 2B). Furthermore, the percentage of [^{3}H]Ro 15–1788 binding sites immunoprecipitated by anti-γ3 antiserum was not increased by inclusion of anti-γ1 antiserum. Again, consistent with the Western blots, γ1 and γ3 do not coexist in the same receptor molecule. For the γ2 and γ3 subunit combination in whole brain, the sum of the percentages of [^{3}H]muscimol binding sites immunoprecipitated by anti-γ2 and -γ3 subunit antisera separately (73%) (Table 2B) was more than that obtained when receptors were immunoprecipitated with anti-γ2 and -γ3 antibodies in combination (66 ± 7%) (Table 2B), although it was within the limit of error of the measurement. For [^{3}H]Ro 15–1788 binding sites, the sum of the percentages of binding sites immunoprecipitated by anti-γ2 and anti-γ3 antisera individually was the same as the percentage immunoprecipitated when the antisera were combined (96%). However, the population of receptors containing a γ3 subunit was small, such that if receptors contained both a γ2 subunit and a γ3 subunit this population could be masked by the errors in the measurement of the number of immunoprecipitated radioligand binding sites. Data from the immunoprecipitation experiments are entirely consistent with at least some γ3-containing receptors existing in combination with γ2 and support the coexistence of these two subunits identified by Western blotting.

Virtually all of the [^{3}H]Ro 15–1788 binding sites (96%) (Table 2B) were immunoprecipited from whole brain by a combination of anti-γ2 and anti-γ3 antibodies. This implies that all GABA$_A$ receptors that bind [^{3}H]Ro 15–1788 contain a γ2 and/or a γ3 subunit. In both cerebellum and whole brain a small proportion of receptors remained that bound [^{3}H]muscimol but were not immunoprecipitated by any of the γ subunit-specific antisera. These could contain a δ subunit in place of a γ subunit or, alternatively, could be composed of α and β subunits only.

Which α and γ subunits coassemble together in a receptor molecule?. There is considerable evidence that in rat brain the γ2 subunit exists in combination with most, if not all, of the α subunits (4, 7, 8, 17). Some of these receptors could also contain a γ3 subunit. However, the composition of γ1-containing receptors has not been studied. Western blot analysis was used to establish which α subunit(s) might occur in the same receptor complex as a γ1 subunit. Receptors were purified on α subunit-specific immunoaffinity resins synthesized using polyclonal antisera raised against the expressed recombinant cytoplasmic loop fragments of α1, α2, α3, α6, and δ subunits, as described (12, 22).[1] Purified material was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the anti-γ1 antiserum (1/500). It was possible to do this only with relatively abundant α subunits, namely α1, α2, α3, and α6. As shown in Fig. 7, of the α subunits investigated only the α2 was identified as a partner for γ1. This observation agrees well with immunoprecipitation studies using GABA$_A$ receptors immunoprecipitated from the cerebellum, where α2 subunits were found to coexist with γ1 (22). It is also in accord with the extensive colocalization of these two subunits in various brain regions (31, 32).

[1] K. Quirk, N. Gillard, P. Whiting, C. I. Ragan, and R. M. McKernan. Receptors containing a δ subunit form a novel class of GABA$_A$ receptors. Manuscript in preparation.

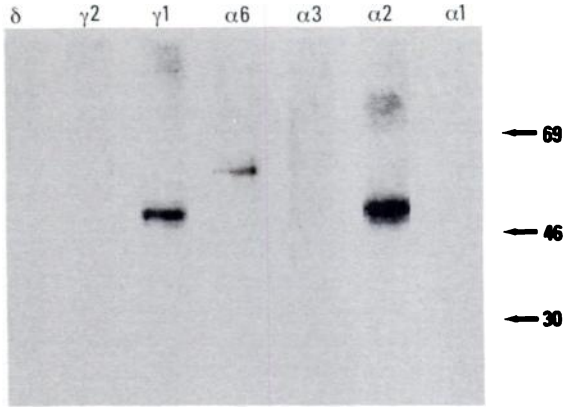

Fig. 7. α2 and γ1 subunits coexist in a receptor molecule. GABA$_A$ receptors were purified on immunoaffinity resins specific for α1, α2, α3, α6, γ1, γ2, and δ subunits and were subjected to SDS-PAGE as indicated. After transfer to nitrocellulose the Western blot was probed with the anti-γ1 antibody (1/500). Approximately 0.5 pmol of receptor was loaded in each lane. The γ1 antibody identified a broad specific band centred at 51 kDa. A nonspecific band was occasionally observed in the α6 lane (at approximately 58 kDa). Molecular size markers (× 1000) are shown on the *right*.

Discussion

Antibodies specific for the γ1, γ2, and γ3 subunits of the GABA$_A$ receptor have been raised by expressing the unique cytoplasmic loop regions in bacteria, purifying them by preparative SDS-PAGE, and raising polyclonal antisera in rabbits. These antibodies can specifically immunoprecipitate receptors containing the respective subunits and, crucially for the experiments presented here, do not show any detectable cross-reactivity. Antiserum raised against a large sequence of the cytoplasmic loop region of the γ2 subunit, γ2(Leu^{317}–Met^{403}), had the highest titer. Three types of data indicated that it did not cross-react with the γ1 subunit. First was its inability to detect the recombinant γ1 polypeptide (Fig. 1). Second was its inability to detect a synthetic γ1 peptide, which contains 17 amino acids common to γ2 (see Materials and Methods). The limit of detection of the synthetic γ1 peptide was 10 μg (3 nmol). This is at least 1000 times more than the amounts of receptor applied to Western blots in these studies, and therefore any signal detected by the anti-γ2 antiserum could not be attributed to the presence of a γ1 sequence. Third was the observation that receptor from rat brain immunopurified on a γ1 affinity resin was not detected on a Western blot by the anti-γ2(Leu^{317}–Met^{403}) antiserum (Fig. 6).

The anti-γ2(Leu^{317}–Met^{403}) antiserum did not cross-react with the γ3 subunit, as evidenced by 1) its inability to detect the expressed γ3 recombinant cytoplasmic loop polypeptide by Western blot and 2) its inability to immunoprecipitate receptors from transfected cells expressing GABA$_A$ receptors of defined composition α1β3γ3. Furthermore, anti-γ2(Leu^{317}–Met^{403}) maximally immunoprecipitated the same proportion of GABA$_A$ receptors as did anti-γ2(Leu^{317}–Tyr^{367}). We concluded therefore that the antisera used here are specific and that the carboxyl-terminal portion (Tyr^{367}–Met^{403}), which contains a stretch of 17 amino acids common to both γ1 and γ2 subunits, is not a major immunogenic region.

From immunoprecipitation studies γ2 was the most abundant of the γ subunits, followed by γ3, with γ1 being the least abundant. This compares reasonably well with the relative

abundance of mRNA indicated by *in situ* hybridization (13, 15, 31–33). Allowing for the observation that some receptors may contain both γ2 and γ3 subunits, up to 84% of all $GABA_A$ receptors contain at least one γ subunit (Table 2B, γ1 plus γ2 plus γ3). It is possible that the remaining receptors are constructed of only α and β subunits or that they contain a δ subunit in place of a γ subunit. We have observed that receptors immunoprecipitated with an antiserum raised to the δ subunit intracellular loop do not bind benzodiazepine site ligands with high affinity (22).[1] The anti-δ subunit antiserum immunoprecipitated 18% of [^{3}H]muscimol binding sites from whole brain, i.e., a large proportion of those remaining after immunoprecipitation with anti-γ1 plus anti-γ2 plus anti-γ3 antisera.

The observation that a receptor can contain more than one γ subunit is an intriguing one, and it raises questions about the stoichiometry and arrangement of subunits in the $GABA_A$ receptor. The ratio of [^{3}H]muscimol to [^{3}H]Ro 15–1788 binding sites for receptors immunoprecipitated by either anti-γ2 or anti-γ3 antiserum is 2.4:1. The most obvious interpretation of this finding is that most naturally occurring receptors have two high affinity binding sites for GABA and one benzodiazepine binding site. A ratio significantly higher than 2:1 could indicate the presence of receptors that do not contain a high affinity benzodiazepine binding site. Receptors that contain a γ2 subunit but do not have high affinity for [^{3}H]Ro 15–1788 exist in the rat brain; these include cerebellar α6βnγ2 (βn is β1, β2, or β3) receptors (22) and presumably receptors that contain both α4 and γ2 (10, 31, 32). Together, these probably represent only a small proportion of the total $GABA_A$ receptor population and their contribution to the γ2-containing population would be minimal, certainly consistent with the interpretation of the binding data representing a stoichiometry of two GABA binding sites and one benzodiazepine binding site per receptor molecule. The ratio of [^{3}H]muscimol to [^{3}H]Ro 15–1788 binding sites for anti-γ3-immunoprecipitated receptors of 1:2.3, which is not significantly different from that for γ2, suggests that, despite the presence of two types of γ subunits (γ2 and γ3) in a significant proportion of the receptors, the receptors still contain only one benzodiazepine binding site. The relative numbers of binding sites for benzodiazepines, GABA agonists, and channel site ligands could be supported by information obtained from receptors of defined composition, such as in a stably transfected (23, 34) or cultured cell line (35). To date, the only data of this sort that have been published are for $GABA_A$ receptors expressed using the baculovirus system, where the GABA to benzodiazepine site ratio was variable, depending on the ratio of subunit constructs introduced into the cells, and maximally reached a ratio of 1:1 (34). In stably transfected L cells expressing α1β1γ2 receptors the ratio of GABA to benzodiazepine binding sites was close to that observed for receptors immunoprecipitated with anti-γ2 or anti-γ3 antisera,[2] and a suggestion that each GABA receptor contains two GABA sites, one benzodiazepine site, and one channel binding site has been made (36). Clearly, additional studies are required to clarify this issue.

Based on experimental evidence to date, comparison with the nicotinic acetylcholine receptor, and consideration of the physicochemical size of the receptor (2, 4, 5, 23), a pentameric structure is suggested. It has been well documented that two α subunits can be present in a receptor (17–21), and it is possible that these may contribute to both the benzodiazepine (29) and GABA binding sites (37, 38). It has also been reported that the β subunit may be the subunit responsible for GABA binding (39). With subunit cooperativity, experimentally observed for the GABA gating of endogenous receptors as a Hill slope of 2 (40–42), it follows that receptors could also contain two β subunits. In this report we have shown that some $GABA_A$ receptors contain more than one γ subunit. Taken together, these observations suggest that no general rule of stoichiometry exists and that different receptor subtypes could have different subunit stoichiometries. There is precedence for this in the nicotinic acetylcholine receptor gene family; the subunit stoichiometry (α1)2β1γδ, which is present at the neuromuscular junction, does not appear to exist in the central nervous system, where the major brain nicotinic receptor subtype has been proposed to have the arrangement (α4)2(β2)3 (43), whereas the ganglionic nicotinic receptor may have the subunit structure α3α5β4, with an as yet unknown stoichiometry (44).

It is difficult to compare the pharmacology of $GABA_A$ receptors immunoprecipitated with different γ subunit-specific antibodies. Benzodiazepine sites on receptors containing a γ1 subunit could not be labeled by any of the currently available ligands, although receptors containing a γ1 subunit clearly have a benzodiazepine binding site, albeit with lower affinity for many ligands (13, 45). Ro 15–4513, zolpidem, and flunitrazepam are all pharmacologically active at γ1-containing receptors expressed in oocytes, but their reduced affinity (13, 45) makes them unsuitable as ligands for the type of binding assay used here, in which bound and free ligand are separated by filtration. [^{3}H]Flunitrazepam has been used to label benzodiazepine sites of $GABA_A$ receptors of composition α1β1γ1 in transfected cells (12), but this could not be reproduced here with naturally occurring receptors of likely composition α2βnγ1. It is possible that, although α1β1γ1 produces a higher affinity benzodiazepine site in transfected cells, this subunit combination does not occur in the central nervous system. This is supported by data presented in Fig. 7, where the γ1 subunit was not detected in receptors purified on an α1 immunoaffinity resin. However, it is not possible to unequivocally rule out the possibility that small populations of receptors exist that contain γ1 in combination with α subunits other than α2.

The pharmacologies of receptors containing γ2 or γ3 subunits are easier to establish in transfected cells where receptors contain only one defined type of α subunit. The multiple pairings of γ2 or γ3 with α subunits precluded an examination of the pharmacology of the benzodiazepine site of receptors immunoprecipitated with γ2 or γ3 subunit-specific antisera. This is consistent with the work of others (20, 46), who have reported pairing of γ2 with α1, α2, and α3, and is also consistent with the pharmacology of anti-α1-, anti-α2-, and anti-α3-immunoprecipitated receptors (17). The pharmacology of γ3-containing receptors has not been well documented, but receptors composed of α1β2γ3 expressed in transfected cells have been reported to show reduced affinity for several benzodiazepines (14). Although the binding of [^{3}H]Ro 15–1788 showed similar affinity for receptors immunoprecipitated with γ2 or γ3 subunit-specific antisera, all other compounds had low Hill slopes and their components could not easily be fitted by a two-site model (data not shown).

We have begun to try to directly address the question of

[2] P. C. Harkness and P. J. Whiting, unpublished observations.

which α subunits and γ subunits are commonly paired *in vivo* and have directly observed that the γ1 subunit is predominantly paired with α2, although the sensitivity of the Western blot protocol used here could not exclude the existence of small populations of other α subunits in combination with γ1. The pairing of γ1 with α2 is consistent with a high ratio of [^{3}H]muscimol to [^{3}H]Ro 15–1788 binding sites observed in receptors immunoprecipitated with anti-α2 antiserum (17), indicating that a proportion of α2-containing receptors do not have a high affinity benzodiazepine binding site. The coassembly of α2 with γ1 is also of interest, because these two genes are localized on the same region of chromosome 4 (47), suggesting that their close proximity might have some functional significance.

In summary, we have demonstrated that receptors exist that contain both γ2 and γ3 subunits and, further, that there may be coordinate, rather than indiscriminate, pairing of α and γ subunits. Further analysis of subunit pairing, including β and δ subunits, should allow a definition of the minimum number of subunit combinations that form *bona fide* GABA$_A$ receptors in the rat brain.

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