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Brain $GABA_A$ Receptors Studied with Subunit-Specific Antibodies

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Abstract

Brain $GABA_A$/benzodiazepine receptors are highly heterogeneous. This heterogeneity is largely derived from the existence of many pentameric combinations of at least 16 different subunits that are differentially expressed in various brain regions and cell types. This molecular heterogeneity leads to binding differences for various ligands, such as GABA agonists and antagonists, benzodiazepine agonists, antagonists, and inverse agonists, steroids, barbiturates, ethanol, and Cl^- channel blockers. Different subunit composition also leads to heterogeneity in the properties of the Cl^- channel (such as conductance and open time); the allosteric interactions among subunits; and signal transduction efficacy between ligand binding and Cl^- channel opening. The study of recombinant receptors expressed in heterologous systems has been very useful for understanding the functional roles of the different $GABA_A$ receptor subunits and the relationships between subunit composition, ligand binding, and Cl^- channel properties. Nevertheless, little is known about the complete subunit composition of the native $GABA_A$ receptors expressed in various brain regions and cell types. Several laboratories, including ours, are using subunit-specific antibodies for dissecting the heterogeneity and subunit composition of native (not reconstituted) brain $GABA_A$ receptors and for revealing the cellular and subcellular distribution of these subunits in the nervous system. These studies are also aimed at understanding the ligand-binding, transduction mechanisms, and channel properties of the various brain $GABA_A$ receptors in relation to synaptic mechanisms and brain function. These studies could be relevant for the discovery and design of new drugs that are selective for some $GABA_A$ receptors and that have fewer side effects.

Index Entries: $GABA_A$ receptor; benzodiazepine receptor; antibody; mRNA; structure.

Introduction

One of the most unexpected findings in the study of the brain $GABA_A$ receptors ($GABA_AR$)/benzodiazepine receptors (BZDR) is their high molecular heterogeneity with regard to subunit composition. The study of receptor subunit combinations expressed in heterologous systems (*Xenopus laevis* oocytes and mammalian or insect cultured cells after mRNA

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injection or DNA transfection) has shown that subunit composition affects the affinity of the $GABA_AR$ for the neurotransmitter GABA and for other ligands and modulators, such as benzodiazepines, barbiturates, steroids, and ethanol. In addition, the properties of the Cl^- channel (i.e., conductance and open time) are affected by the subunit composition.

A major challenge in the field is the identification of all the different $GABA_AR$ that are present in the brain, the cells that express particular subunit combinations, and the subcellular compartments (i.e., axon vs soma vs dendrites vs specific synapses) where the different $GABA_AR$ are located *(1–3)*. Recent experiments with single-cell reverse transcriptase polymerase chain reaction (RT-PCR) and electrophysiological measurements indicate that different subunit combinations can be assembled by a single neuron into various functionally distinguishable $GABA_AR$, at least in primary cultures *(3)*. Light and electron microscopy (EM) immunocytochemistry experiments in brain support the existence of different subunit combinations in different cell compartments *(1,2)*.

The expression of subunit combinations in heterologous systems has provided crucial information about the subunits required for the assembly of functional $GABA_AR/Cl^-$ channels having various modulatory sites. One limitation of studying recombinant receptors is that it is not known how well they represent the native receptors expressed by brain neurons.

Recently our laboratory has elucidated for the first time the pentameric subunit composition of a native cerebellar receptor using subunit-specific antibodies (*see* Subunit Composition of Native Receptors). This subunit combination has not yet been studied in heterologous systems. Many of the subunit combinations present in brain have not been reconstituted in vitro, since the number of cloned subunits from the mammalian brain is at least 16 (*see* next section) combining into pentameric hetero-oligomers *(4)*. Under these circumstances, several hundred thousand pentameric subunit combinations are theoretically possible *(6)*. We do not know how many combinations occur in brain. Thus, we are now at the stage in which the identification of the subunit composition of the native $GABA_AR$ in various brain regions and cell types is needed. Our laboratory is involved in making and using subunit-specific antibodies in this kind of study.

There are important aspects of the $GABA_AR$ that have been covered by others in recent reviews, such as the biochemistry, cloning, and expression in heterologous systems for studying the relationship between subunit composition, ligand binding, and rCl^- channel properties *(5–9,130,134)*. In addition, there are several comprehensive studies on the mapping of subunit mRNAs in the mammalian brain and spinal cord by *in situ* hybridization *(10–13)*. I refer the reader to these reviews and studies for detailed coverage of the subjects. In this article I will review the progress made in our laboratory and in others' studying the native (brain) $GABA_AR$ by using subunit-specific antibodies.

The Heterogeneous Brain $GABA_AR/BZDR$

In 1987, Schofield et al. *(14)* published the cloning and the first complete amino-acid sequences of the ~~1 and BX subunits of the bovine $GABA_AR$. Since then, several subunit classes and isoforms within each class have been cloned in the mammalian brain: α_1–α_6, β_1–β_3, γ_1–γ_3, δ, ρ_1–ρ_2 *(5,8,15)*. The different subunits are encoded by different genes. Amino-acid identity among isoforms of the same class is ~70% whereas the identity among classes is ~30%. All subunits have similar membrane topology, having a long N-end extracellular peptide, four α helix putative transmembrane domains (M1–M4), a short extracellular C-end peptide, and a large intracellular loop (IL) between M3 and M4 *(14)*. Additional heterogeneity is shown by the γ_2 subunit, which is expressed as γ_{2s} (short) and γ_{2L} (long) forms resulting from alternative RNA splicing

(16,17). The γ_{2L} is identical to the γ_{2S} except for the insertion in the former of an octapeptide that is encoded by a 24-base exon. The octapeptide has a consensus phosphorylation amino-acid sequence for protein kinase C located in the IL expanding between the putative transmembrane domains M3 and M4. Alternative exon splicing has been predicted for the human β_3 gene, which would result in two mRNAs that differ in the signal peptide *(18)*. Alternative splicing of α_6 RNA affecting the extracellular N-end peptide has also been described. Only the most abundant form of α_6 (the long form) is involved in the formation of functional receptors *(19)*. Additional subunits (β_4 and γ_4) and spliced forms (β_{2L}, β_{4S}, β_{4L}) have been found in chicken but not in mammals *(20–22)*.

The native GABA$_A$R are pentamers *(4)*. It is not yet known how many pentameric combinations exist in native brain receptors as indicated above, but it is clear that native brain receptors show a good deal of heterogeneity, as binding and biochemical studies have shown. The CL218–872, some β-carbolines, and zolpidem can differentiate several types of brain GABA$_A$R/BZDR (types I, II$_M$, and II$_L$) with different affinities for these compounds *(23,24)*. In addition, photoaffinity labeling of GABA$_A$R with [^{3}H]flunitrazepam (FNZ) has revealed (after sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and flurography) several photolabeled GABA$_A$R subunits. This technique has been used to show that these subunits are differently expressed in various brain regions *(25,26)* and during brain development *(23,27)*. It is thought that the photoaffinity labeled peptides of 51,000, 53,000, 57,000, and 59,000 Mr *(23)* are the α_1, α_2, α_5, and α_3 subunits, respectively, based on their mobility in SDS-PAGE as determined by immunoblotting with subunit-specific antibodies (*see* Biochemical Studies with Subunit Specific Antibodies). Further heterogeneity of the GABA$_A$R was revealed by the binding of the partial inverse agonist [^{3}H]Ro15-4513. This ligand binds to two different GABA$_A$R types *(28–32)*. One type binds diazepam and clonazepam with high affinity, making the binding of [^{3}H]Ro15-4513 diazepam (or clonazepam) sensitive (DZS). The other receptor type does not bind diazepam or clonazepam with high affinity and therefore the binding of [^{3}H]Ro15-4513 is diazepam (or clonazepam) insensitive (DZI). We know that in the cerebellum, the DZI Ro15-4513 binding site corresponds to GABA$_A$R having the α_6 subunit, whereas the DZS site corresponds to receptors containing α_1, and perhaps some having α_2, α_3, or α_5 *(28,33–35)*.

Additional biochemical evidence for heterogeneity of native receptors was derived from studies on affinity purified receptors by three different methods:

1. Affinity chromatography on immobilized Ro7-1986/1 *(36,37)*;
2. Affinity chromatography on immobilized 1012-S *(38)*; and
3. Immunoaffinity chromatography on monoclonal antibody (MAb) 62-3G1 *(39–41)*.

Although the three methods yielded similar GABA$_A$R/BZDR, there were some differences in the subunit composition and binding properties. We also found differences in the subunit composition and benzodiazepine binding between the immunoaffinity-purified receptors from cortex and cerebellum *(41)*. More recently, immunopurifications with subunit-specific antibodies have shown a great deal of heterogeneity in native GABA$_A$R (*see* Immunocytochemical Studies with Subunit-Specific Antibodies; Subunit Composition of Native Receptors).

In situ hybridization results are also consistent with the existence of high molecular heterogeneity in brain GABA$_A$R, since the expression of the different subunit mRNAs vary through the brain and cell types *(10–13)*. In addition, some neuronal types express many subunit mRNAs. The extreme case is represented by the granule cells of the dentate gyrus, which express all GABA$_A$R subunit mRNAs with the exception of α_6. Nevertheless, the expression of a subunit mRNA does not guarantee its translation into a peptide nor its

assembly into mature $GABA_AR$. The studies with subunit-specific antibodies described below have allowed us and others to address these issues. Another limitation of *in situ* hybridization studies is that when they reveal, for example, that cerebellar granule cells express α_1, α_6, β_2, β_3, γ_2, and δ, we do not know whether each individual granule cell expresses all the subunits or whether single cells differ from each other in subunit expression. Some of these questions recently have been answered by single-cell RT-PCR experiments that have shown that in culture, some cerebellar granule cells express α_1 but not α_6 mRNAs, others express α_6 but not α_1, and others express both α_1 and α_6. The same study has shown by electrophysiological recording of the same cultured cells that not all cells assemble the same functional type of $GABA_AR$ and that a single cell can assemble more than one type of $GABA_AR$ *(3)*.

Subunit-Specific Antibodies to the $GABA_AR$

The first subunit-specific antibodies to the $GABA_AR$ were MAbs prepared by Hans Möhler's group, then at Hoffmann La Roche, Inc. *(42)*, by our group, then at the University of New York at Stony Brook *(43)*, and by John Tallman's group, then at Yale University *(44)*. These MAbs were obtained by immunizing mice with bovine brain $GABA_AR$ purified by affinity chromatography on Ro7-1986/1 as indicated above. Several MAbs immunoprecipitated the brain $GABA_AR$, however, only three of them are still widely used in the literature for immunocytochemistry. These are the MAb 62-3G1 to $\beta_{2/3}$ *(37,45)*, bd17 to $\beta_{2/3}$, and bd24 to α_1 *(42,46)*. The amino-acid epitopes that these MAbs recognize have been determined *(47,48)*. All are located extracellularly near the amino terminus of the corresponding subunit. We have recently made mouse MAbs to the γ_2 subunit *(49)*.

Following the cloning of the $GABA_AR$ subunits, and therefore after deducing the amino-acid sequences, several groups, including ours, have prepared subunit-specific antibodies by immunizing rabbits and other animals with synthetic peptides coupled to carrier proteins. Following this approach, our group has obtained specific antisera to each of the described mammalian $GABA_AR$ subunits *(34,35,50–54,129,* and unpublished results). The antisera immunoprecipitated the population of brain $GABA_AR$ that contained the corresponding subunit. In addition, we have also prepared specific antisera for γ_{2S} or γ_{2L} *(13, 50–52, 54)*. Other groups have also prepared anti-synthetic peptide antibodies to various $GABA_AR$ subunits *(28,55–70)*.

The cloning of the cDNAs has also allowed us to express different regions of the various subunits as bacterial fusion proteins. We have also made subunit-specific antisera by immunizing rabbits with the fusion proteins. In this way we have prepared fusion proteins and antibodies to the IL of α_1, β_1, β_2, β_3, γ_{2S}, and δ *(32,34,54,71,72,* and unpublished results). We have selected the IL, which expands between M3 and M4, because computer analysis shows little identity among the amino-acid sequences of the IL of various subunits *(5)*. We have also produced MAbs to the human γ_{2S} subunits by immunizing mice with the γ_{2S} IL fusion protein *(49)*. All our antifusion protein antibodies also immunoprecipitated the corresponding population of native $GABA_AR$. Others have also produced antisera (not monoclonal antibodies) to fusion proteins of the IL of several $GABA_AR$ subunits *(68,73–76)*.

Immunocytochemical Studies with Subunit-Specific Antibodies

We and others have extensively used our MAb 62-3G1 for revealing the distribution of $GABA_AR$ in brain, spinal cord, and retina by both light microscopy and EM immunocytochemistry (Fig. 1. and refs. *43,45,50–53,71,77–86*). Although our MAb 62-3G1 recognizes both β_2 and β_3 subunits, the results have provided an extensive mapping of the $GABA_AR$ since β_2

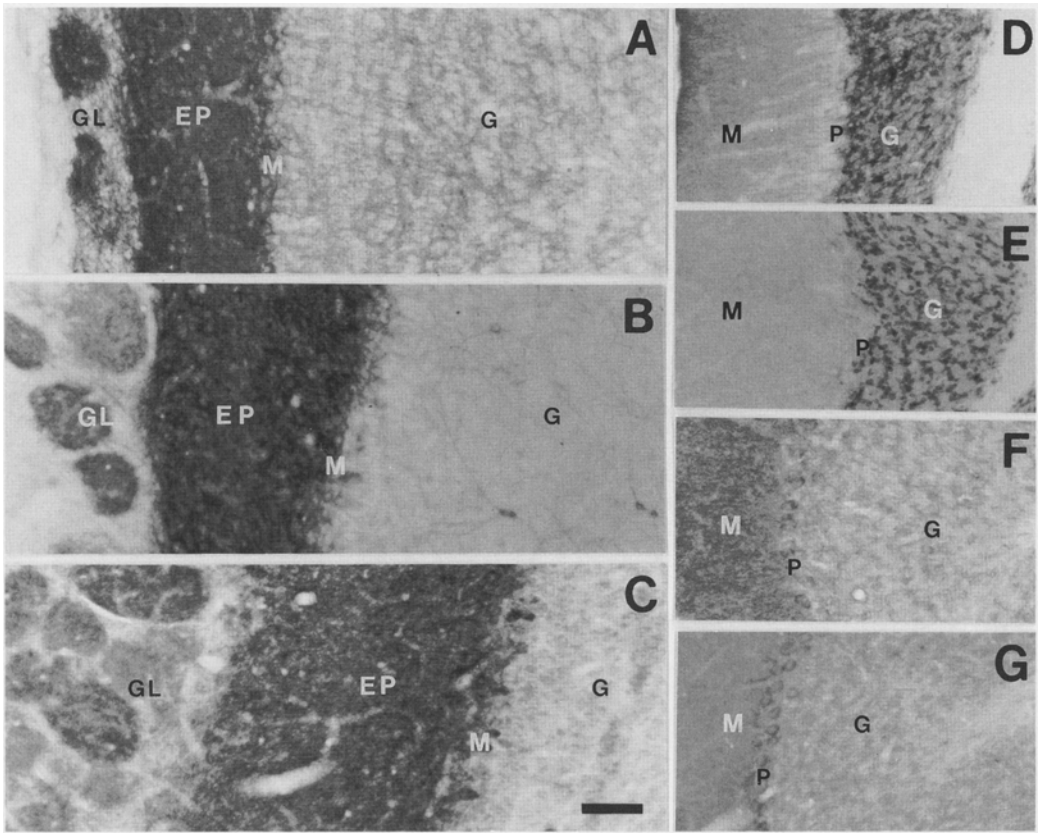

Fig. 1. Rat brain immunocytochemistry with some of the anti-GABA$_A$ receptor antibodies in the olfactory bulb **(A–C)** and cerebellum **(D–G)** with MAb 62-3G1 to $\beta_{2/3}$ (A,D), anti-β_2IL (B,E), anti-α_1COOH (C), anti-γ_{2S} (F), and anti-γ_{2L} (G). EP, external plexiform layer; G, granule cell layer; GL, glomeruli; M, mitral cell layer (A–C) or molecular layer (D–G); P, Purkinje cell layer. Parasagittal sections. Scale bar = 77 μm.

and/or β_3 subunits are present in many GABA$_A$R *(32,34,59,66,71,72)*. These results have revealed the high presence of GABA$_A$R in many GABAergic synapses, including both the pre- and postsynaptic membranes. In addition, many neuronal GABA$_A$R are also located extrasynaptically. There are also GABA$_A$R subunits that are localized in intracellular compartments, probably revealing subunits in the process of synthesis, assembly, and/or degradation.

The MAb 62-3G1 has also been used to study the developmental expression of $\beta_{2/3}$ peptides in different brain areas and species. These include the sensory motor cortex of the macaque monkey *(84)*, rat neocortex *(87)*, and rat thalamus *(88)*. These studies have revealed developmental changes in the laminar distribution of the $\beta_{2/3}$ subunits. The results have also shown the presence of GABA$_A$R in the brain subplate, which supports the notion that transient GABAergic synaptic connectivity occurs in the subplate. In other cortical layers, the expression of the receptors precedes the axogenesis of cortical neurons and the formation of symmetrical synapses. Therefore, the results are consistent with a morphogenetic role of GABA acting through GABA$_A$R during early embryogenesis. The MAb 62-3G1 has also been used to show that the density of GABA$_A$R in the monkey visual cortex (areas 17 and 18) is regulated not only by denervation of the ocular imput but also by reduced electrical activity *(82,83)*. The results support

the notion that decreased GABAergic activity in the cortical columns driven by the deprived eye might contribute to the functional expansion of the ocular dominance columns driven by the other eye.

The MAbs bd17 to $\beta_{2/3}$ and bd24 to bovine and human α_1 have also been extensively used in light and EM immunocytochemistry for $GABA_AR$ localization *(1,46,89,90,133)*, developmental studies *(91)*, and studies on synaptic plasticity *(83)*. The bd17 has been more frequently used than bd24 because the latter shows species-specificity and it does not react with the rat α_1 subunit. The immunocytochemical results obtained with our MAb 62-3G1 and Möhler's bd 17 are very similar, which is expected from two antibodies that seem to recognize similar, if not the same epitope *(48)*.

We have also obtained excellent immunocytochemical results with an affinity purified anti-β_2 rabbit antibody to a β_2IL fusion protein *(71)*. The comparative immunocytochemistry (Fig. 1) between the MAb 62-3G1 to $\beta_{2/3}$ and the anti-β_2IL has allowed us not only to reveal the brain distribution of β_2 but also to identify neurons that express β_3 but not β_2 *(71)*. At the present time, we are trying to develop good immunocytochemical reagents for β_1 and β_3 subunits from our already generated antisera to β_1IL and β_3IL fusion proteins, respectively. An anti-α_6IL antibody and an anti-α_1 peptide antibody made in McKernan's and Sieghart's laboratories, respectively, have been successfully used in EM immunocytochemistry *(1,133)*.

I should indicate here that polyclonal antisera to either coupled synthetic peptides or fusion proteins need to be affinity purified on immobilized antigen before they can be used for immunocytochemistry. Otherwise, the nonspecific reactivity of the antiserum with the brain tissue produces unacceptable levels of background. Even affinity-purified antibodies to coupled peptides are frequently unsuitable for immunocytochemistry. Only trial and error and the review of the literature on the successes with antipeptide antibodies to the $GABA_AR$ and to other receptors of the same gene family allows the rational design of strategies for selecting the right peptide sequences for immunizations. It is desirable that the antibodies to peptides also recognize the native $GABA_AR$. Some antipeptide antibodies do not immunoprecipitate native receptors. This is probably because of the selection of peptide sequences located either in a highly structured receptor epitope or in a region of the native $GABA_AR$ that is inaccessible to the antibody (a hidden epitope) for steric hindrance or other reasons. Even when the antipeptide antibodies immunoprecipitate the native detergent-solubilized receptors, they might not be good enough for immunocytochemistry because:

1. The antibody also reacts with epitopes present in other proteins, giving a high nonspecific background;
2. The antibody cannot bind to the receptor in its membrane environment (which increases the steric hindrance and antibody penetration problems); and
3. The epitope is denatured by the aldehyde fixation required by the immunocytochemical techniques.

Thus, immunocytochemistry imposes the most severe test for antibody use. Recent advances regarding the development of mild embedding procedures allow the preservation of native epitopes that were lost after more traditional embedding procedures. These developments are allowing the use of post-embedding immunogold methods for the study of $GABA_AR$ subunit colocalization by EM immunocytochemistry *(90,133)*.

We have successfully used several of our affinity-purified antibodies to α_1, γ_1, γ_{2S}, γ_{2L}, γ_3, α_4, and α_6 synthetic peptides for mapping the localization of the corresponding subunit in the rat brain by light microscopy immunocytochemistry (Fig. 1 and refs. *50–53,129*, and unpublished results). At the present time we are also aiming to develop antipeptide antisera to other subunits valuable for immunocytochemistry. Others have also used light microscopy immunocytochemistry with various antipeptide antibodies (to α_1, α_2, α_3, α_5, α_6, β_1, β_2, γ_1, γ_2, and δ) for studying the distribution of

$GABA_AR$ subunits in various brain regions *(59,60,64,66,92–95,132)* and during brain development *(69,91,96,97)*. Double and triple immunofluorescence immunocytochemistry has also been used to study the colocalization of some receptor subunits in the same neuron. These studies have revealed colocalization of $\alpha_1\,\beta_2\,\gamma_2$; $\alpha_1\,\beta_{2/3}\,\gamma_2$; $\alpha_2\,\beta_3\,\gamma_2$; $\alpha_2\,\beta_{2/3}\,\gamma_2$; $\alpha_3\,\beta_{2/3}\,\gamma_2$; $\alpha_5\,\beta_{2/3}\,\gamma_2$; $\alpha_1\,\alpha_2\,\gamma_2$; $\alpha_1\,\alpha_3\,\beta_{2/3}\,\gamma_2$; $\alpha_2\,\gamma_2$; $\alpha_3\,\gamma_2$; $\alpha_1\,\alpha_3\,\gamma_2$; $\alpha_2\,\alpha_3\,\gamma_2$; and $\alpha_2\,\alpha_5\delta$ *(2,66,132)*. Double immunofluorescence studies have also shown that most serotonergic neurons express α_3 but not α_1, whereas many GABAergic neuron can express α_1 or α_3 *(98,99)*. Subunit colocalization in the same protein cluster has been studied by freeze-fracture EM immunocytochemistry using antipeptide and MAbs *(100)*. We should indicate that the immunocytochemistry studies do not have the resolution required to show subunit colocalization in a single $GABA_AR$ molecule. The availability of antibodies to various peptide epitopes in combination with EM immunogold immunocytochemistry will allow the study of the location of the epitopes relative to the membrane (extracellular vs intracellular face), which will test the current topology model for subunit insertion in the membrane (with four transmembrane domains) presently based on hydropathy profiles *(14)*.

There is good agreement between the results obtained with immunocytochemistry and *in situ* hybridization regarding receptor subunit distribution. Nevertheless, the results are not entirely identical, which is expected given the different localization of mRNAs (mostly in the cell somas) and the localization of the subunit peptides (which can be inserted in the cell membranes and concentrated at distal synapses). It is also possible that not all the expressed subunit mRNAs are always translated into protein. Another factor to consider is that the subunit-specific antibodies can recognize not only mature and fully assembled membrane receptors, but also unassembled subunits as well as subunits in the process of synthesis and degradation that occurs in intracellular compartments.

Biochemical Studies with Subunit-Specific Antibodies

We have used our MAb 62-3G1 in immunoaffinity chromatography experiments for the purification of $GABA_AR$ from bovine cerebral cortex and cerebellum that contain $\beta_{2/3}$ subunits *(39–41)*. The MAb 62-3G1 recognized in immunoblots two peptide bands of 55 and 57 kDa both in rat and bovine brain. These results are consistent with the immunoblot results obtained with two antifusion protein antibodies also made in our laboratory. The antibody to β_2IL fusion protein reacted with two peptides of 55 and 57 kDa of rat brain membranes *(71)*, whereas anti-β_3IL fusion protein only reacted with a 57 kDa peptide *(72)*. Benke et al. *(66)*, using antipeptide antibodies to β_1, β_2, and β_3, have also reported that the anti-β_2 antibody recognizes two peptides of 54 and 56 kDa, whereas anti-β_3 and anti-β_1 recognize peptides of 57 kDa. Immunoblot experiments using other antipeptide antibodies to β subunits have also revealed immunoreactive bands in the 54–58 kDa range *(101–103)*.

We have made three mouse MAbs and two rabbit antisera to γ_2IL fusion proteins and two antipeptide antisera specific for γ_{2S} or γ_{2L}. Immunoblots show that all the anti-γ_2IL antibodies react with a wide peptide band of 44–49 kDa, whereas anti-γ_{2S} and anti-γ_{2L} react with 45 and 47 peptides, respectively, *(32,49,54)*. Anti-γ_1 and γ_3 peptide antibodies, also made in our laboratory, react with 47 and 44 kDa peptides, respectively *(129)*. These results show that all the γ subunits have similar mobility in SDS-PAGE. Other groups using different antipeptide antibodies have reported similar Mr for γ_2 (43–49 kDa; *58,59,69*), γ_1 (45–51 kDa; *68*), and γ_3 (43–46 kDa; *70*).

Our anti-α_1, anti-α_4, and anti-α_6 antipeptide antibodies react with 51, 66, and 58 kDa peptides, respectively (*34,35*, and unpublished results). Other authors using antipeptide or antifusion protein antibodies have shown that α_1 is 50–51 kDa *(61,63,65,73,102)*, α_2 is 52–53 kDa *(61,63,73,102)*, α_3 is 58–61 kDa *(61,65,73, 102)*, α_4 is 67 kDa *(67)*, α_5 is 53–55 kDa *(65,73)*, and α_6

Table 1
The Mr of the Mammalian $GABA_AR$ Subunits Determined by Immunoblotting with Subunit-Specific Antibodies

Subunit	Mr × 10^{-3}	
	1[a]	2
α_1	51	50–51
α_2	—	52–53
α_3	—	58–61
α_4	66	67
α_5	—	53–55
α_6	58	57–58
β_1	—	57
β_2	55 and 57	54 and 56
β_3	57	57
γ_1	47	45–51
γ_2	44–49	43–49
γ_{2S}	45	—
γ_{2L}	47	—
γ_3	44	43–46
δ	—	54

[a]1: Mr values determined in our laboratory with our antibodies; 2: Mr values determined in other laboratories with other antibodies as indicated in the text. —: Not determined.

is 57–58 kDa *(28,76,97)*. The δ subunit has been reported to be 54 kDa *(60)*. Table 1 shows the molecular mass of the $GABA_AR$ subunits revealed with immunoblotting experiments.

$GABA_AR$ Immunoprecipitation with Subunit-Specific Antibodies

The $GABA_AR$ immunoprecipitation with subunit-specific antibodies is being done for four main purposes:

1. To calculate the proportion of $GABA_AR$ that have a particular subunit;
2. To study the ligand-binding specificities of the $GABA_AR$ that contain a particular subunit;
3. To study colocalization of different subunits in the same receptor; and
4. To elucidate the subunit composition of native receptors.

Two conditions need to be fulfilled for an antibody to be valuable in these studies: The antibody must react well with the native solubilized $GABA_AR$ and it should not interfere with the binding of various ligands to the $GABA_AR$.

Quantification of $GABA_AR$ Subunit Peptides in Various Brain Regions

We have already discussed the use of immunocytochemical techniques for the localization of $GABA_AR$ subunits in various CNS regions, cell types, and subcellular compartments. This level of resolution cannot be achieved by immunoprecipitation. However, immunoprecipitation of radioligand binding activity can be accurately quantified. Moreover, this technique quantifies mature and fully assembled $GABA_AR$, whereas immunocytochemistry can reveal not only the assembled subunits but also the unassembled ones and subunits in the process of synthesis and degradation as indicated above.

The first immunoprecipitation studies with subunit-specific antibodies were done with the MAbs to α_1 and $\beta_{2/3}$, *(37,42)*. Immunoprecipitation with other subunit-specific antibodies were done as they were developed by several groups, including ours. Results are emerging although it is not always easy to compare the data obtained by different groups, since immunoprecipitations have been done using different brain regions (i.e., whole brain vs cerebral cortex vs cerebellum), different receptor preparations (solubilized crude membranes vs affinity-purified receptors, or brains from different species, such as rat, cow, and pig), and different radioligands (muscimol, FNZ, Ro15-1788, and so forth). We know that the affinity for each ligand depends on the $GABA_AR$ subunit composition. In addition, not all reported immunoprecipitations are quantitative. We *(32,34,49,54,71,72)* and others *(104)* have found the need for two or three sequential immunoprecipitations with the same antibody for reaching the maximum (quantitative) immunoprecipitation of detergent solubilized $GABA_AR$ from crude membrane preparations. However, quantitative immunoprecipitation of affinity-purified receptors can be well accom-

Table 2
Immunoprecipitation of Rat Brain GABA$_A$R with Subunit-Specific Antibodies

	% Immunoprecipitation					
	Cerebral cortex		Cerebellum			
				[^{3}H]Ro15-4513		
Antibody to[a]	[^{3}H]Muscimol	[^{3}H]FNZ	[^{3}H]Muscimol	[^{3}H]FNZ	DZS[b]	DZI[b]
α_1COOH	—[c]	70	76	96	87	51
α_1IL	—	80	94	—	94	76
α_6	—	—	39	—	18	81
β_2IL	93	93	—	—	—	—
β_3IL	61	68	—	50	—	—
$\beta_{2/3}$ MAb 62-3G1	90	78	84	96	72	65
γ_1	22	—	19	—	—	—
γ_2IL	73	89	—	—	—	—
γ_2IL MAb KC5-E51	66	89	—	—	—	—
γ_2	64	—	—	—	—	—
γ_{2S}	42	52	34	31	39	66
γ_{2L}	27	37	47	65	51	40
γ_3	16	—	18	—	—	—

[a]These results were obtained in our laboratory with our antisera *(see text)*. All are antisera except the MAb 62-3G1 to $\beta_{2/3}$ and MAb KC5-E51 to γ_2IL.

[b]DZS and DZI refer to the diazepam sensitive and diazepam insensitive components of [^{3}H]Ro15-4513 binding (*see text* and refs. *32, 34, 35,* and *54*).

[c]—: Not determined.

plished with only one immunoprecipitation under optimal conditions. Immunoprecipitation values also depend on the capability of the antibody to recognize well the native solubilized receptors and on the specificity of the antibody for a particular subunit vs the others (i.e., absence of crossreactivity). By selecting specific amino-acid sequences, antipeptide antibodies can be made totally subunit-specific. Unfortunately, specific antipeptide antibodies do not always bind well to the native receptors, as discussed above. On the other hand, we have found that antisera to IL fusion proteins recognize very well the native receptors but they can crossreact with the large IL of other isoforms of the same subunit class (i.e., γ_1, γ_2, and γ_3) since the isoforms of the same class share some conserved amino-acid sequences in the IL *(32,49,71,72)*. However, antisera to the IL of a particular subunit do not crossreact with isoforms from other classes.

Table 2 shows a summary of the quantitative immunoprecipitation values obtained in our laboratory with various subunit-specific antibodies. With MAb 62-3G1, which only recognizes β_2 and β_3, we could immunoprecipitate 88–93% of [^{3}H]muscimol binding and 73–83% of [^{3}H]FNZ binding in the rat cerebral cortex and 84% of [^{3}H]muscimol and 96% of [^{3}H]FNZ binding in the cerebellum *(32,49,54,72)*. In the hippocampus and olfactory bulb, the same antibody immunoprecipitated 86 and 96% of [^{3}H]FNZ binding, respectively *(72)*. These results show the abundant presence of the β_2 and/or β_3 subunits in the brain GABA$_A$R. High immunoprecipitation values of [^{3}H]muscimol and [^{3}H]FNZ were also obtained in cerebral cortex with antibodies to the large IL of β_2 (93%) and β_3 (61–68%) *(71,72)*, although, as we have mentioned above, these antibodies probably have some crossreactivity with the IL of other β subunits.

Two antibodies to the α_1 subunit also made in our laboratory, one to the carboxy-end (anti-α_1COOH) and another one to the IL of α_1 (anti-α_1IL), immunoprecipitate 70 and 80%,

respectively, of the [^{3}H]FNZ binding from rat cerebral cortex. In cerebellum they precipitate 76 and 94% of [^{3}H]muscimol binding, respectively. Thus, the immunoprecipitation results show the ubiquitous presence of the α_1 subunit in brain $GABA_AR$. In addition, the anti-α_1COOH precipitates 51% or the diazepam insensitive DZI-[^{3}H]Ro15-4513 binding to the cerebellar $GABA_AR$. This result indicates that about 50% of the cerebellar α_6-containing receptors also have the α_1 subunit, since the DZI-[^{3}H]Ro15-4513 binding is characteristic of α_6-containing receptors (*see* next section). We have also done immunoprecipitations with an anti-α_6 antibody, which also suggests the existence of coassembly of α_1 and α_6 in the same cerebellar receptor as discussed below. Coassembly of α_1 and α_6 has also been reported by others *(28,111,124,125)*. Nevertheless, one group has found no evidence for α_1 and α_6 coassembly *(75)*.

Two antibodies to the γ_2IL fusion proteins (anti-γ_2IL1 and anti-γ_2IL2), and three antipeptide antibodies (anti-γ_2, anti-γ_{2S}, and anti-γ_{2L}) specific for γ_2 (recognizing both γ_{2S} and γ_{2L}), γ_{2S}, and γ_{2L}, respectively, were made in our laboratory. In addition, we have also prepared three MAbs to γ_2IL. The anti-γ_2IL1 and anti-γ_2IL2 precipitate 70–76% of [^{3}H]muscimol and 87–91% of [^{3}H]FNZ binding of rat cerebral cortex membranes *(32,54)*. The anti-γ_2 peptide immunoprecipitated 64% of [^{3}H]muscimol binding. The three MAbs to γ_2IL (KC5-E51, KC4-8A7, and KC4-2G7) immunoprecipitated 56–66% of [^{3}H]muscimol binding and 79–89% of [^{3}H]FNZ binding *(49)*. Anti-γ_{2S} and anti-γ_{2L} immunoprecipitated 42 and 27% of [^{3}H]muscimol and 52 and 37% of [^{3}H]FNZ binding, respectively, showing the predominance of γ_{2S} over γ_{2L} in the cerebral cortex. The relative abundance of γ_{2S} and γ_{2L} varies in different brain regions. Thus, the ratio γ_{2S}/γ_{2L} goes in the following order: olfactory bulb (6.3) > hippocampus (1.85) > cerebral cortex (1.54) > cerebellum (0.45) > inferior colliculus (0.24) *(13,54)*. These results are in good agreement with the quantitative distribution of γ_{2S} and γ_{2L} mRNAs by *in situ*, Northern blot, and dot blot hybridizations *(13)*. The immunoprecipitation studies have shown that α_1, $\beta_{2/3}$, and γ_2 are the most abundant $GABA_AR$ subunits through the brain and that they are present in many $GABA_AR$, frequently colocalizing in the same receptor molecule. Thus, we have shown that α_1, $\beta_{2/3}$, and γ_2 coexist in 50% of the $GABA_AR$ of the rat cerebral cortex. These results agree well with *in situ* hybridization studies that have also shown that the most abundant $GABA_AR$ subunit mRNAs in the brain are α_1, β_2, β_3, and γ_2 *(10–12)*.

Other groups have also done $GABA_AR$ immunoprecipitations with subunit-specific antibodies to quantify the presence of various subunits in brain $GABA_AR$ *(58–61,63,65,66, 73,75,76)*. Table 3 includes immunoprecipitation values obtained by other groups using other subunit-specific antibodies. For comparative purposes with Table 2, Table 3 only includes immunoprecipitation studies using solubilized $GABA_AR$ from rat brain membranes. These studies have also shown the high abundance of α_1, β_2, and γ_2 subunits throughout the brain and that α_1, $\beta_{2/3}$, and γ_2 frequently colocalize in the same $GABA_AR$ molecule *(58,59,66)*.

Benzodiazepine Binding and Subunit Composition of Native Receptors

Most of the relationships between benzodiazepine binding properties and subunit composition are derived from recombinant receptors expressed in heterologous systems. Moreover, site-directed mutagenesis studies have revealed the importance of several amino acids present in various subunits for the binding of GABA and benzodiazepines. These studies have shown that high-affinity GABA binding is mainly provided by the β subunits, although the α and γ subunit also contribute to it *(105–107,128,131,134)*. Reconstituted receptors with combinations of the three main subunit classes (α, β, and γ) are similar to the brain counterparts in terms of binding properties, allosteric interactions, and Cl^- channel function. High-affinity benzodiazepine binding is

Table 3
Immunoprecipitation of Rat Brain GABA$_A$R with Subunit-Specific Antibodies

	% Immunoprecipitation					
	Whole brain		Cerebral cortex		Cerebellum	
	[^{3}H]Muscimol	[^{3}H]Ro15-1788	[^{3}H]Muscimol	[^{3}H]Ro15-1788	[^{3}H]Muscimol	[^{3}H]Ro15-1788
α_1	—	81,[a] 40[b]	—	79[e]	70,[g] 30[h]	95,[e] 90[h]
α_2	—	28[h]	—	—	11[h]	4[h]
α_3	—	18,[a] 24[b]	—	—	12[h]	13[h]
α_5	—	10[a]	—	14[a]	—	—
α_6	—	—	—	—	25,[g] 56[h]	—
β_1	—	8[c]	—	—	—	—
β_2	—	57[c]	—	—	—	—
β_3	—	14[c]	—	—	—	—
γ_1	11[d]	0[d]	—	—	9[d,h]	0[d]
γ_2	59[d]	78[d]	—	—	68,[d] 63[h]	108[d]
γ_3	14[d]	18[d]	—	—	—	—
δ	—	30[a]	—	17[f]	23[h]	0,[h] 23[f]

The results were obtained in several laboratories with antisera different from the ones used in Table 2. —: Not determined.
[a]Mertens et al. *(65)*.
[b]McKernan et al. *(73)*.
[c]Benke et al. *(66)*.
[d]Quirk et al. *(76)*.
[e]Benke et al. *(138)*.
[f]Benke et al. *(60)*.
[g]Lüddens et al. *(28)*.
[h]Quirk et al. *(75)*.

mainly provided by the α subunits, although the γ subunits are also needed for this binding as well as for the allosteric benzodiazepine stimulation of the GABA induced Cl^- channel opening *(108–112,135)*. Receptors reconstituted with the γ_2 subunit (plus α and β) show high-affinity binding of benzodiazepine agonists, antagonists, and inverse agonists. Nevertheless, there are reports indicating that the γ_2 subunit in combination with either α or β subunits is all that is required for a GABA$_A$ receptor to be sensitive to BZDs *(105,113,114)*. The GABA$_A$R reconstituted with γ_3 subunits (plus α and β) also show high-affinity binding for benzodiazepine agonists, antagonists, and inverse agonists although the affinity for agonists is two orders of magnitude smaller for γ_3 than γ_2 *(115)*. The γ_1 also produces reconstituted receptors with one or two orders of magnitude lower affinity for benzodiazepine agonists and with many orders lower for antagonists and inverse agonists than the ones reconstituted with γ_2 *(116)*. Table 4 shows the relationship between subunit presence and benzodiazepine binding to recombinant GABA$_A$R.

The reconstitution approach has also been very useful for understanding the relationship between subunit composition and particular benzodiazepine pharmacology. Thus, type-1 BZDR (with high-affinity for zolpidem, CL218-872, and some β carbolines) is associated with the presence of the α_1 subunit whereas reconstitution with α_2, α_3, or α_5 produces type-II BZDR *(73,117–120)*. In addition, GABA$_A$R reconstituted with α_5 show lower affinity for zolpidem than the ones reconstituted with α_2 or α_3 *(121)*. A single amino-acid change from Glu to Gly at position 225 of α_3 increases the affinity of this subunit for CL21-872 10-fold. In addition, α_1 has gly 200 in the homologous position. Therefore, this Gly seems to be involved in type-I BZDR pharmacology *(109)*. Reconstituted receptor studies have also

Table 4
Relationship Between Subunits and Benzodiazepine Binding Affinities of Recombinant $GABA_AR$[a]

	K_D range (M)						
	Flunitrazepam	Flumazenil	β-Carboline			Ro15-4513	
Subunit	(agonist)	(antagonist)	(inverse agonist)	CL 218-872	Zolpidem	DZS	DZI
α_1	10^{-9}	10^{-9}	10^{-9}	10^{-7}	10^{-8}	10^{-9}	$>10^{-5}$
α_2	10^{-9}	10^{-9}	10^{-9}	10^{-6}	10^{-7}	10^{-9}	$>10^{-5}$
α_3	10^{-9}	10^{-9}	10^{-9}	10^{-6}	10^{-7}	10^{-9}	$>10^{-5}$
α_4	$>10^{-5}$	10^{-7}	10^{-7}	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	10^{-9}
α_5	10^{-9}	10^{-8}	10^{-9}	10^{-7}	$>10^{-5}$	10^{-9}	$>10^{-5}$
α_6	10^{-5}	10^{-7}	10^{-7}	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	10^{-9}
γ_1	10^{-8}	$>10^{-5}$	$>10^{-5}$	?[b]	10^{-8}	?	?
γ_2	10^{-9}	10^{-9}	10^{-9}	10^{-7}–10^{-6}	10^{-8}–10^{-7}	10^{-9}	10^{-9}
γ_3	10^{-7}	10^{-9}	10^{-9}	10^{-8}	10^{-6}–10^{-5}	10^{-9}	$>10^{-5}$

[a]These results were obtained by coexpressing each of the α subunits with β_2 and γ_2 and each of the γ subunits with α_1 (or α_2 or α_3) and β_2 (or β_1 or β_3). Data are from refs. *28,115–117,121,136,137,139.*
[b]?: Unreported.

shown that the DZI binding of Ro15-4513 is associated with α_6 or α_4 whereas α_1, α_2, α_3, and α_5 show DZS binding of Ro15-4513. The presence of His 101 in α_1, α_2, and in the homologous position in α_3 and α_5 is necessary for the Ro15-4513 binding to be DZS *(122)*. This His is missing in α_4 or replaced by Arg in α_6. Besides His 101 and Gly 200 in α_1, Thr 162 and Val 212 (and the homologous amino acids in the other α subunits) are also involved in benzodiazepine binding *(33)*. The Thr 142 from the γ_2 subunit also seems to participate in the binding of benzodiazepines to the $GABA_AR$ *(112)*. Recombinant receptors containing either α_4 or α_6 (plus β_2 and γ_2) neither bind (with high affinity) diazepam nor other benzodiazepine agonists nor β-carbolines *(28)*. Recombinant receptors have also been used to study the contribution of α, β, and γ subunits to the binding of GABA agonists *(123)*.

We and others have used subunit-specific antibodies to study the relationship between benzodiazepine binding and subunit composition of brain receptors. Anti-α_1 antibodies immunoprecipitate type-I BZDR whereas anti-α_2 and anti-α_3 immunoprecipitate type-II *(65,66,73,118,119)*. Moreover, we have found that all the type-I BZDR in the rat cerebral cortex have α_1, that 75% of these receptors also have γ_2 and $\beta_{2/3}$, and 20–25% of the receptors have α_1 but have neither γ_2 nor $\beta_{2/3}$ *(119,120)*. We have also shown that anti-α_6 and anti-α_1 antibodies immunoprecipitate the DZI and the DZS-[^{3}H]Ro15-4513 binding, respectively, to the cerebellar receptors *(34,35)*. Togel et al. *(70)* have shown that the brain $GABA_AR$ immunopurified with anti-γ_3 have about two orders of magnitude less affinity for FNZ and zolpidem (benzodiazepine agonists) than $GABA_AR$ immunoprecipitated with anti-γ_2, whereas the affinities for antagonists and inverse agonists were similar *(70,76)*. The brain $GABA_AR$ immunopurified with anti-γ_1 show low affinity for both flumazenil *(68)* and Ro15-4513 *(75,76)*. Thus, the binding properties of the studied immunoprecipitated brain receptors agree well with the data obtained with reconstituted receptors. Nevertheless, given the high heterogeneity in subunit composition of the brain $GABA_AR$, we should not expect to always find agreement between the binding properties of the immunoprecipitated native receptors and the ones reconstituted with two or three different subunits. No immunoprecipitation studies have addressed yet the contribution of the various subunits to the steroid, barbiturate, alcohol, or other modulatory binding sites.

Subunit Composition of Native Receptors

The elucidation of the various pentameric subunit combinations of the native $GABA_AR$ is not an easy task given the large heterogeneity of the $GABA_AR$ known to exist in the brain as indicated above. Expression in heterologous systems indicate that the main properties of the native $GABA_AR$/BZDR can be reconstituted with a combination (likely pentameric) of α, β, and γ subunits (i.e., $\alpha_1\ \beta_2\ \gamma_2$). We and others have also shown that the α_1, $\beta_{2/3}$, and γ_2 subunits coexist in many native $GABA_AR$ receptors *(32,34,58,59,66)*. Several groups have studied the colocalization of subunit pairs in brain $GABA_AR$ by subunit-specific immunopurification of the $GABA_AR$ followed by immunoblotting *(34,35,54,58,61,65,66,68,70, 73,75,76,101,124)*. The following combination pairs have been reported to be present in the brain $GABA_AR$: $\alpha_1\ \alpha_2$; $\alpha_1\ \alpha_3$; $\alpha_1\ \alpha_6$; $\alpha_2\ \alpha_3$; $\alpha_1\ \gamma_2$; $\alpha_1\ \gamma_3$; $\alpha_2\ \gamma_1$; $\alpha_2\ \gamma_3$; $\alpha_3\ \gamma_3$; $\alpha_4\ \gamma_3$; $\alpha_6\ \gamma_3$; $\alpha_1\ \beta_2$; $\alpha_1\ \beta_3$; $\alpha_2\ \beta_3$; $\alpha_3\ \beta_3$; $\gamma_{2S}\ \gamma_{2L}$; $\gamma_2\ \gamma_3$; $\gamma_3\ \beta_{2/3}$; $\alpha_1\ \delta$; $\alpha_3\ \delta$; $\delta\ \beta_{2/3}$. These results show the extensive molecular heterogeneity of the brain $GABA_AR$.

Nevertheless, none of these studies have revealed the complete pentameric subunit composition of any brain $GABA_AR$. Recently, by using a combination of quantitative immunoprecipitation, immunoaffinity purification, and specific radioligand binding assays we have elucidated the pentameric subunit composition of a cerebellar receptor: $\alpha_1\ \alpha_6\ \gamma_{2S}\ \gamma_{2L}\ \beta_{2/3}$ *(34)*. This was the first time that the complete pentameric composition of a brain receptor has been reported in the literature. This subunit composition is consistent with recent data showing the coexistence of two γ subunits *(54,75)* or two α subunits *(34,61,63,65,111,124–126)* in some $GABA_AR$. Moreover, Backus et al. *(127)* have shown that in host cells, functional receptors are formed by preferred pentameric combinations that include two α, two γ, and one β subunits. Recombinant receptors of the complexity of the aforementioned native cerebellar receptor have not yet been studied in heterologous expression systems. These native cerebellar receptors formed by $\alpha_1\ \alpha_6\ \gamma_{2S}\ \gamma_{2L}$ and $\beta_{2/3}$ have two classes of benzodiazepine binding sites; one contributed by α_1 and the other one contributed by α_6, as we have recently shown *(35)*. The α_1 site has DZS-[^{3}H]Ro15-4513 binding, whereas the α_6 site shows DZI-[^{3}H]Ro15-4513 binding. Thus, in the $GABA_AR$ that contain both α_1 and α_6, each α subunit maintains its individual benzodiazepine binding characteristics. We do not know yet whether this is a common feature of all the $GABA_AR$ that combine two other α subunits *(61,63,65,121)*. The receptors that have two α subunits might also require two γ subunits for high-affinity binding of benzodiazepines to each of the α subunits and for the highest efficacy in the BZD modification of the GABA induced Cl^- channel opening. The aforementioned cerebellar receptors have two α and two γ_2 subunits. It is conceivable that receptors having two different α subunits, but only one γ_2 subunit, have a single benzodiazepine binding site. This site would be present on the α subunit that is in proper contact with the γ_2 subunit. We have already indicated that the γ_2 subunit is necessary for the α subunit to bind benzodiazepines *(131,134)*. Moreover, the asymmetry of each subunit might lead to one benzodiazepine binding site even if the two α subunits are in contact with the only γ_2 subunit that is present in the pentamer. In the aforementioned experiments, Backus et al. *(127)* has also shown that two other functional pentameric combinations (two α, two β, and one γ as well as one α, two β, and two γ) are also formed, although they are less likely to assemble than the combination of two α, two γ, and one β. Therefore, the prediction derived from this additional molecular heterogeneity is that the relative number of GABA and benzodiazepine binding sites, allosteric interactions among subunits, and the efficacy of signal transduction in the $GABA_AR$ not only depends on the subunits present in the pentamer but also on the stoichiometry and relative position of the subunits. One of the present challenges in this field is to elucidate and understand in functional terms such extensive molecular heterogeneity of brain $GABA_AR$. Subunit-specific antibodies will play a major role in these studies.

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