Assembly and Heterogeneity of $GABA_A$ Receptors

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$GABA_A$ receptors ($GABA_A$Rs) are pentameric, ligand-gated chloride channels that mediate the majority of fast inhibitory synaptic neurotransmission in the brain. The receptors are assembled from a repertoire of 19 subunits (α1-6, β1-3, γ1-3, δ, ε, π, and ρ1-3), providing the possibility for vast isoform heterogeneity. Because the subunit subtypes included in a receptor determine its physiological and pharmacological properties, identification of receptor isoforms has clear clinical relevance. A large body of literature indicates that $GABA_A$Rs do not assemble randomly; rather, incorporation of specific subunits into a receptor is regulated at many levels. Each subunit has a characteristic temporal and spatial expression pattern; however, most neurons express many $GABA_A$R subunits at once. Consequently, certain "rules" of assembly must exist to limit receptor heterogeneity. In this review, we discuss the regulation of $GABA_A$R biogenesis, including limitation of heterogeneity, as well as the specific receptor isoforms that have been identified *in vivo.*

Phasic inhibition
Inhibition resulting from transient activation of synaptic $GABA_A$ receptors by presynaptically-released GABA; gives rise to inhibitory postsynaptic currents (IPSCs).

Tonic inhibition
Inhibition resulting from persistent activation of peri- or extrasynaptic $GABA_A$ receptors by ambient GABA.

Benzodiazepines
Compounds that potentiate the response of certain $GABA_A$ receptors; used clinically for their anticonvulsant, anxiolytic, sedative, and amnestic effects.

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The vast majority of inhibitory neurotransmission in the brain is mediated by γ-aminobutyric acid (GABA). It has been detected in approximately 30% of all synapses[1] and acts via ionotropic $GABA_A$ receptors, which mediate fast inhibitory neurotransmission[2], and metabotropic $GABA_B$ receptors, which mediate slower inhibitory effects[3]. $GABA_A$ receptors ($GABA_A$Rs) are chloride channels belonging to the Cys-loop receptor superfamily of ligand-gated ion channels (LGIC), which also includes nicotinic acetylcholine receptors (nAChR), 5-hydroxytryptamine type 3 receptors (5-HT3), and glycine receptors (GlyR)[4]. Like most members of this superfamily, $GABA_A$Rs are pentamers that are assembled from an array of homologous subunits. All subunits share a common structure: each contains a large, extracellular N-terminal domain, which contains the ligand-binding site and the eponymous Cys-loop; four α-helical transmembrane domains (M1-4); a large intracellular loop between the third and fourth transmembrane helices (M3-M4 loop); and a very short, extracellular C-terminal domain[5] (**Figure 1a**).

Nineteen subunits, grouped by sequence homology into eight subunit families, have been identified for the $GABA_A$ receptor: α1-6, β1-3, γ1-3, δ, ε, π, and ρ1-3[6]. Several of these subunit subtypes also undergo alternative splicing and/or RNA editing, further increasing the potential diversity of $GABA_A$ receptor isoforms. Each subunit exhibits a characteristic expression pattern in the brain; however, these patterns overlap extensively. Indeed, a single neuron can express many subunits simultaneously. Consequently, many but not all of the mathematically-possible $GABA_A$R isoforms could exist somewhere in the brain. The most common isoforms, however, are thought to comprise two α subunits, two β subunits, and one γ or δ subunit[7-9] (**Figure 1b**), though this remains a subject of vigorous debate.

The large variety of $GABA_A$R isoforms exhibit a concomitant variety of physiological properties[2]. For instance, most receptors containing a γ subunit are located in the synapse, where they mediate phasic inhibition in response to presynaptically-released GABA[10]. These receptors have a relatively low affinity for GABA, activate quickly, desensitize extensively, and deactivate slowly. Conversely, receptors containing a δ subunit are located outside the synapse, where they mediate tonic inhibition in response to low concentrations of ambient GABA. Unsurprisingly, δ-subunit-containing receptors also differ physiologically; they have a relatively high affinity for GABA, activate slowly, desensitize minimally, and deactivate rapidly[11].

Additionally, $GABA_A$Rs have been linked to many diseases and disorders, including epilepsy[12-14], insomnia[15], anxiety[16], depression[16], schizophrenia[17], alcoholism[18], and autism[19]. Predictably, then, $GABA_A$Rs are targeted by numerous drugs, particularly sedatives, anxiolytics, and anticonvulsants; examples include benzodiazepines, zolpidem, etomidate, and propofol[20, 21]. Both the pathology and the pharmacology of $GABA_A$Rs depend highly upon receptor subunit composition – for instance, epilepsy-associated mutations have been identified only in the α1, β3, γ2, and δ subunits, and benzodiazepines act only at receptor isoforms containing both a γ subunit and certain α subunits.

Given the prevalence of $GABA_A$R expression, the pathology resulting from receptor malfunction, and the pharmacological dependence upon isoform

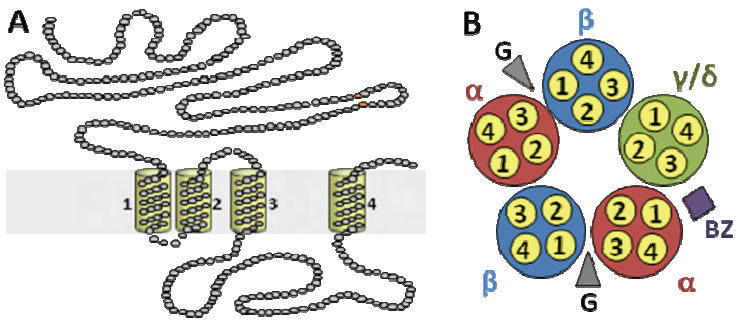

Figure 1 | **$GABA_A$ receptor morphology. a |** Structure of a $GABA_AR$ subunit. Cys-loop cysteines marked in orange; transmembrane domains enclosed in cylinders and numbered 1-4. **b |** Schematic view of most common $GABA_AR$ isoform (putative) from the synaptic cleft . G = GABA binding site; BZ = benzodiazepine binding site.

Zolpidem
Compound with structure and physiological effects similar to those of benzodiazepines; used clinically to treat insomnia.

Etomidate and propofol
Intravenous general anesthetics that potentiate the response of certain $GABA_A$ receptors.

N-linked glycosylation
The transfer, by the ER-resident enzyme oligosaccharyl transferase, of a 14-sugar "core" oligosaccharide to asparagines on newly-synthesized polypeptides. Asparagines contained in the sequence Asn-Xaa-Ser/Thr (where Xaa is any amino acid other than proline) are candidates for glycosylation.

Glycan trimming
The modification of core oligosaccharides by enzymes in the Golgi apparatus.

Palmitoylation
The covalent attachment of palmitate, a 16-carbon saturated fatty acid, to cysteine residues.

identity, it is clearly important to understand the process of receptor assembly. Therefore, in this review, we will examine the generation of $GABA_AR$ diversity. First, we will review the general processes of receptor biogenesis, after which we will discuss the selective oligomerization of $GABA_AR$ subunits. Finally, we will examine the ultimate product of these processes: native $GABA_A$ receptor isoforms.

BIOGENESIS OF $GABA_A$ RECEPTORS

As with other LGICs, $GABA_A$ receptor subunits are inserted co-translationally into the membrane of the endoplasmic reticulum (ER). There, they fold and oligomerize in a process that depends heavily upon ER-resident chaperones. The process of receptor oligomerization is slow and inefficient; studies suggest that approximately 70% of subunits are degraded without being incorporated into a pentameric receptor, and receptors do not appear on the cell surface for several hours following transfection[22]. While in the ER, $GABA_A$ receptor subunits also undergo typical protein modifications, including the early stages of N-linked glycosylation. Interestingly, however, N-linked glycosylation is not required for subsequent forward trafficking, although multiple glycosylation sites have been identified on all subunits[23] and glycosylation is necessary for proper assembly and trafficking of other Cys-loop receptors[24, 25]. Properly folded and assembled subunits proceed to the Golgi apparatus, where they undergo further modification such as palmitoylation and glycan trimming[26]. With the assistance of multiple $GABA_AR$-associated proteins, receptors are then trafficked to the neuronal surface. They may be inserted directly into their final subcellular location (*i.e.* post-, peri-, or extrasynaptic), or they may diffuse into that location after membrane insertion[27]. Finally, $GABA_ARs$ undergo constitutive and activity-dependent endocytosis (both clathrin-dependent and clathrin-independent)[28], after which they are recycled to the cell surface or targeted for lysosomal degradation. Every step of $GABA_A$ receptor assembly and trafficking is regulated by signals within the subunits[29] as well as by various associated proteins[30].

SELECTIVE OLIGOMERIZATION OF $GABA_A$ RECEPTOR SUBUNITS

After temporal and spatial regulation of subunit expression, the first (and, arguably, the most important) opportunity for a neuron to control what $GABA_A$ receptor isoforms it will produce is the process of selective subunit oligomerization. Presumably, a neuron expressing many $GABA_AR$ subunit subtypes would have a hierarchical yet flexible assembly mechanism that favors association between certain subunits and, ultimately, directs the incorporation of assembly intermediates (e.g. dimers, trimers) into full receptors. Indeed, several studies have indicated that, though all subunit combinations can form oligomers, only a subset can form pentamers[23]. This is a key distinction because pentamers are trafficked to the cell surface, but oligomers of lower molecular weight are retained in the ER and subsequently degraded[23, 31]. Importantly, some disease-causing mutations appeared to reduce surface expression and function by disrupting the process of oligomerization[14].

Expression of recombinant subunits in heterologous cells has provided insight into the "rules" governing assembly of the most prevalent subunit subtypes. When expressed individually, α1, β2, and γ2 subunits formed primarily monomers and dimers, as did combinations of γ2 with either α1 or β2/3. Conversely, co-expression of α1 and β2/3 subunits, with or without γ2 subunits, predominantly yielded pentamers, indicating that the combination of α and β subunits is necessary and sufficient for complete receptor assembly[31, 32]. Interestingly, however, receptors including a third (non-α/β) subunit appear to assemble more efficiently. When α, β, and a third subunit (either γ, δ, ε, or π) were co-expressed in heterologous systems, the kinetic signature of αβ receptors could not be detected[33-35]; furthermore, that signature has been detected in very few neurons[36, 37]. Clearly, both neurons and heterologous cells are capable of selective oligomerization, suggesting the existence of assembly signals within the subunits themselves.

Several studies have, in fact, isolated amino acid sequences and individual residues that are important for specific subunit interactions[29, 38]. These sequences have been identified in the α1[39-43], α6[39], β3[42-45], γ2[42, 46], and γ3[47] subunits, primarily in the large N-terminal domain, though there were some reports of assembly sequences in the M3-M4 loop[48, 49]. Although homology modeling based on the

nAChR[50] and AChBP[51] has provided some insight into the structural basis of these interactions, it is important to note that these sequences might not directly contact adjacent subunits; rather, they might simply facilitate oligomerization by encouraging proper protein folding.

HETEROGENEITY *IN VIVO*: NATIVE $GABA_A$ RECEPTOR ISOFORMS

Most studies mentioned thus far have been conducted in heterologous expression systems or in cultured neurons. Because of the great potential for $GABA_AR$ heterogeneity, it is necessary to use such systems to investigate properties of specific subunits (*i.e.* assembly sequences) and isoforms (*i.e.* kinetic and pharmacological properties). Unfortunately, these studies cannot answer a crucial question: what $GABA_A$ receptor isoforms actually exist in the brain? In an attempt to construct a standardized response to that question, the International Union of Pharmacology recently established a list of potential native $GABA_AR$ oligomers[6]. These receptor isoforms were divided into three categories ("identified", "existence with high probability", and "tentative") based on multiple types of evidence. The authors also specified a logical strategy, summarized below, for determining whether or not a receptor isoform exists *in vivo*. First, the long list of potential isoforms can be narrowed based on subunit co-expression patterns, which can be ascertained by *in situ* hybridization and immunoreactivity. If subunits are indeed co-expressed in a specific cell type, evidence for association of those subunits should then be sought, primarily through co-immunoprecipitation. Subunits that associate should be co-expressed in heterologous systems, where electrophysiology can be performed and characteristic kinetics and pharmacology can be assessed. These characteristic properties can then be sought in neurons. Finally, knockout animals can be created and studied for the absence of characteristic physiology and pharmacology associated with isoforms containing the deleted subunit. The list of "identified" and "high probability" isoforms, along with their localization (regional and subcellular) and basic forms of inhibition (phasic or tonic), is presented in **Table 1**.

Isoforms that have been unequivocally identified

Given the widespread distribution of the α1β2γ2 $GABA_AR$ isoform, it is perhaps unsurprising that this isoform is thought to account for up to 60% of all $GABA_A$ receptors in the brain[20]. Mice lacking either the α1 or β2 subunit have been generated; in both lines, total $GABA_AR$ expression in the brain was reduced by more than 50%[52]. A γ2 knockout mouse was found to lack 94% of all benzodiazepine binding sites[53] (recall that the BZ binding site is located at the interface of an α and a γ subunit; consequently, this result indicates that receptors including the γ1 or γ3 subunit might make up only 6% of all αβγ receptors). As indicated in **Table 1**, the other five α subunits can likewise co-assemble with β and γ2 subunits. Strong evidence for the existence of these αxβxγ2 receptors is provided by isoform-specific pharmacology from benzodiazepine (BZ) site ligands. Such ligands include classic benzodiazepines (*i.e.* diazepam); imidazobenzodiazepines (*i.e.* flumazenil and Ro15-4513); and the so-called "Z-drugs" (*i.e.* zolpidem and zaleplon).

Classic benzodiazepines cannot bind receptors containing α4 or α6 subunits, and they have much lower affinity for receptors containing γ1 or γ3 subunits than for receptors containing γ2 subunits. Furthermore, through the use of transgenic mice, the various actions of benzodiazepines have been attributed to specific α subunit subtypes. Point mutations conferring diazepam insensitivity were introduced into the genes of individual α subunits and the resulting mice were subjected to behavioral tests with and without administration of diazepam[54, 74, 75]. Results indicated that the α1 subunit mediated the sedative, anterograde amnestic, and some of the anticonvulsant effects of diazepam[74, 76]; the α2 and α3 subunits mediated the anxiolytic and muscle-relaxant effects[54, 75] and the α5 subunit was involved in amnestic effects as well as other aspects of learning and memory. Imidazobenzodiazepines, however, bind without regard to α subunit subtype. Therefore, receptors that are benzodiazepine-insensitive but imidazobenzodiazepine-sensitive can be identified as α4βγ2 or α6βγ2 isoforms. Conversely, Z-drugs act with differing potency at BZ-sensitive isoforms containing α1,2,3, or 5; specifically, they display high potency at α1βγ2 isoforms, lower potency at α2βγ2 and α3βγ2 isoforms, and no action at α5βγ2[77]. Taken together, these pharmacological properties allow positive identification of α1βγ2 and α5βγ2 receptors, as well as tentative identification of α(2,3)βγ2 and α(4,6)βγ2 receptors; however, expression patterns can differentiate these latter two pairs of isoforms. Consequently, all αβγ2 isoforms are considered to have been identified *in vivo*.

The aforementioned evidence accounts for six of the 11 identified native isoforms. Four of the remaining five isoforms contain the δ subunit, which possesses many unusual properties that help to identify δ-subunit-containing isoforms *in vivo*. First, the δ subunit has been found exclusively in extrasynaptic membranes, where it is incorporated into receptors that have a high affinity for GABA and mediate a constant, "tonic" current with low amplitude and little desensitization[11, 78]. The pharmacology of δ-subunit-containing receptors is

Table 1 | **$GABA_AR$ isoforms likely to exist *in vivo*.**

	Areas of high expression	Subcellular localization	Type of inhibition	Refs
Identified				
α1β2γ2	cerebral cortex (all layers) hippocampus (interneurons, principal cells) thalamus (relay nuclei) cerebellum (Purkinje and granule cells)	synaptic, extrasynaptic	phasic, tonic	52
α2βγ2	cerebral cortex (layers I-IV) hippocampus (pyramidal cells) striatum hypothalamus motor neurons	synaptic (most), extrasynaptic	phasic, tonic	54
α3βγ2	cerebral cortex (layers V-VI) hippocampus thalamus (nRT) cerebellum	synaptic (most), extrasynaptic	phasic, tonic	54
α4βγ2	hippocampus (granule cells) thalamus (relay nuclei)	synaptic, extrasynaptic	phasic, tonic	55
α4β2δ	thalamus (relay nuclei)	extrasynaptic	tonic	55, 56
α4β3δ	dentate gyrus (granule cells); thalamus	extrasynaptic	tonic	55
α5βγ2	hippocampus (pyramidal cells)	extrasynaptic – clustered (minor synaptic population)	tonic	57
α6βγ2	cerebellum (granule cells)	extrasynaptic	phasic	58, 59
α6β2δ	cerebellum (granule cells)	extrasynaptic	tonic	58-60
α6β3δ	cerebellum (granule cells)	extrasynaptic	tonic	58-60
ρ	retina (bipolar cells)	synaptic, extrasynaptic?	tonic?	61-63
Existence with high probability				
α1β3γ2	cortex? hippocampus?	synaptic?	phasic?	6, 64
α1βδ	hippocampus (interneurons)	extrasynaptic	tonic	65
α5β3γ2	hippocampus (pyramidal cells, granule cells)	extrasynaptic	tonic	66
αβ1γ/ αβ1δ	cerebral cortex	?	?	67-69
αβ	hippocampus (pyramidal cells)	extrasynaptic	tonic	36, 37
α1α6βγ/ α1α6βδ	cerebellum (granule cells)	synaptic/extrasynaptic	phasic	58, 60

List of isoforms from reference 6, which also identifies "tentative" isoforms that assembled in heterologous systems (ρ1-3, αβγ1, αβγ3, αβε, αβθ, αβπ, and αxαγβγ2). Also see the following general references: *in situ* hybridization[70]; immunohistochemistry[71, 72]; reviews[20, 73] .

also very different from that of γ-subunit-containing receptors. Though GABA binds to δ-containing isoforms with high affinity, its efficacy is relatively low. Conversely, ethanol[79] and neuroactive steroids[80] act strongly at δ-subunit-containing receptors. Demonstration of these properties *in vivo*[56], combined with co-localization, co-immunoprecipitation, and gene deletion studies[81], have allowed identification of the δ-subunit-containing receptors listed in **Table 1**[55].

The last isoform that has been identified unequivocally *in vivo* comprises ρ subunits alone. These receptors, previously classified as $GABA_C$ receptors due to their unique pharmacology, are expressed predominantly in retinal bipolar cells[63]; however, low levels of ρ subunit transcripts have also been detected in hippocampus[82], cerebellum[83], amygdala[84], and certain brain areas important for visual signal processing (superior colliculus, lateral geniculate nucleus, and visual cortex)[62, 83] . Evidence for both homomeric and heteromeric ρ isoforms has been reported[85, 86]; consequently, the subunit subtypes present in these receptors remain undefined.

Isoforms that exist with high probability

Finally, we will briefly discuss the evidence supporting the "existence with high probability" of certain key $GABA_AR$ isoforms listed in **Table 1**. Each of these isoforms assembles efficiently and has been studied extensively in heterologous systems[11, 31, 33, 35, 80, 87-89]; moreover, the subunits are co-expressed *in vivo*[70-72]. Indeed, most were not classified as "identified" simply because few animal studies have been conducted. First, although α1 and γ2 subunits seem to partner most frequently with the β2 subunit, expression patterns indicate that this cannot always be the case, because certain areas expressing the α1 and γ2 subunits do not express the β2 subunit[71]. In these areas, it is quite likely that α1β3γ2 receptors are formed, as indicated by various pharmacological properties[64]. The evidence supporting the existence of α5β3γ2 is also extensive; the only reason that it is not considered to be unequivocally identified is that, to date, α5 and β3 have not been co-immunoprecipitated[6]. However, these three subunits have been co-localized[71], α5 and β3 subunits were co-depleted in knockout mice[6], α5-selective etomidate effects have been identified[90], and electrophysiology indicates that this isoform mediates tonic inhibition in the hippocampus[66]. Another widely-accepted isoform, α1βδ, clearly assembled in heterologous systems and responded to known modulators of δ-subunit-containing receptors. Furthermore, one recent report identified this isoform in molecular layer interneurons of the hippocampus[65]. Finally, as previously mentioned, two different αβ isoforms have been identified in rat brain via sequential co-immunoprecipitation[37] and electrophysiology[36].

CONCLUDING REMARKS

$GABA_A$ receptors in the brain are ubiquitous, implicated in many diseases, and highly heterogeneous. Each receptor isoform exhibits unique physiological and pharmacological properties and a characteristic expression pattern. Consequently, a thorough understanding of $GABA_AR$ assembly, trafficking, and function could yield significant therapeutic advantages, such as isoform-specific drugs that minimize unwanted side effects. Currently, only 11 $GABA_AR$ isoforms have been conclusively identified *in vivo*, and the existence of another six is considered to be highly probable. Further study of the assembly, trafficking, and function of these receptors may improve clinical practice, as will attempts to identify other $GABA_AR$ isoforms that occur in the brain.

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FURTHER INFORMATION

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