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Pharmrev Fast Forward. Published on September 12, 2008 as DOI:10.1124/pr.108.00505

0031-6997/08/0000-000–000$20.00
PHARMACOLOGICAL REVIEWS Vol. 00, No. 0
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505/3388524
Pharmacol Rev 00:000–000, 2008 *Printed in U.S.A.*

International Union of Pharmacology. LXX. Subtypes of γ-Aminobutyric Acid$_A$ Receptors: Classification on the Basis of Subunit Composition, Pharmacology, and Function. Update

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***Abstract*——In this review we attempt to summarize experimental evidence on the existence of defined native GABA$_A$ receptor subtypes and to produce a list of receptors that actually seem to exist according to current knowledge. This will serve to update the most recent classification of GABA$_A$ receptors (*Pharmacol Rev* 50:291–313, 1998) approved by the Nomenclature Committee of the International Union of Pharmacology. GABA$_A$ receptors are chloride channels that mediate the major form of fast inhibitory neurotransmission in the central nervous system. They are members of the Cys-loop pentameric ligand-gated ion channel (LGIC) superfamily and share struc-**

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tural and functional homology with other members of that family. $GABA_A$ receptors are assembled from a family of 19 homologous subunit gene products and form numerous, mostly hetero-oligomeric, pentamers. Such receptor subtypes with properties that depend on subunit composition vary in topography and ontogeny, in cellular and subcellular localization, in their role in brain circuits and behaviors, in their mechanisms of regulation, and in their pharmacology. We propose several criteria, which can be applied to all the members of the LGIC superfamily, for including a receptor subtype on a list of native hetero-oligomeric subtypes. With these criteria, we develop a working $GABA_A$ receptor list, which currently includes 26 members, but will undoubtedly be modified and grow as information expands. The list is divided into three categories of native receptor subtypes: "identified," "existence with high probability," and "tentative."

I. Introduction: Definition of $GABA_A$ Receptors

γ-Aminobutyric acid (GABA[1]), the major inhibitory neurotransmitter in the brain, exerts its action via ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ receptors ($GABA_A$-Rs) are the major inhibitory receptors in the central nervous system (CNS). They were first identified pharmacologically as being activated by GABA and the selective agonist muscimol, blocked by bicuculline and picrotoxin, and modulated by benzodiazepines, barbiturates, and certain other CNS depressants (Macdonald and Olsen, 1994; Sieghart, 1995). $GABA_B$ receptors are activated by GABA and the selective agonist baclofen but are not sensitive to typical $GABA_A$-R ligands, such as bicuculline and muscimol. $GABA_B$ receptors couple to several different effector systems, depending on the associated G protein (e.g., $G_i/_o$), such as activation of inwardly rectifying K^+ channels or inhibition of high voltage-activated Ca^{2+} channels, involving regulation of cyclic AMP or inositol phosphate signaling (Bowery et al., 2002; Bettler et al., 2004).

The objective of this review is to define $GABA_A$-Rs and, in so doing, to summarize classifications and provide guidelines on nomenclature. This should serve to update the nomenclature suggested by Barnard et al. (1998), which remains useful and relevant, for most issues. $GABA_A$-Rs are chloride channels that are gated by GABA and mediate rapid phasic inhibitory synaptic transmission and also tonic inhibition by producing current in extrasynaptic and perisynaptic locations (Mody and Pearce, 2004; Farrant and Nusser, 2005). They are abundant in the nervous system of all organisms with a nervous system, including invertebrates (Buckingham et al., 2005), which also express $GABA_A$-Rs on some muscle cells (Robinson and Olsen, 1988), where they also mediate Cl^--dependent inhibition. $GABA_A$-Rs are found, but in a limited capacity, in non-neural tissues [such as the pancreas (Borboni et al., 1994)], where their functional roles are still under study and their pharmacological relevance remains to be established. Consistent with the emphasis of the Nomenclature Committee of the International Union of Pharmacology (IUPHAR), the receptors considered here are limited to mammalian species, with emphasis on humans. Because of their widespread localization throughout the mammalian nervous system, $GABA_A$-Rs play a major role in virtually all brain physiological functions and serve as targets of numerous classes of drugs, used both clinically and important as research tools. The $GABA_A$-Rs are a family of probably many receptor subtypes, but so far only a few dozen subtypes have been identified with reasonable certainty. Because of recent advances in knowledge of their molecular makeup, identification of native subunit compositions, and relevance to pharmacological specificity, an update of the list of $GABA_A$-Rs is in order. This list is necessarily a continually changing work in progress.

A. Ionotropic GABA-Gated Anion Channels

Mammalian $GABA_A$-Rs are all anion-selective channels. Increased chloride permeability generally reduces neuronal excitability (inhibition), because the Cl^- equilibrium potential in most mature neurons is near the resting membrane potential and the concentration of chloride within the neuron ($[Cl^-]_i$) is much less than that within the extracellular fluid ($[Cl^-]_o$) (Martin and Olsen, 2000). However, depending on expression of Cl^- transporters, $[Cl^-]_i$ can increase, leading to a Cl^- equilibrium potential that is less negative than the resting membrane potential. Under such conditions, activation of $GABA_A$-Rs can cause membrane depolarization, possibly sufficient to elicit action potential discharge (excitation). The situation occurs in nature and is especially relevant in early development (Ben-Ari, 2002). In addition, on strong activation of $GABA_A$-Rs, the resulting

[1] Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$-R, GABA type A receptor; CNS, central nervous system; IUPHAR, International Union of Pharmacology; LGIC, ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; GlyR, glycine receptor; 5-HT, serotonin; $5HT_3R$, 5-HT_3 receptor; FRET, fluorescence resonance energy transfer; RT-PCR, reverse transcriptase-polymerase chain reaction; flumazenil, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; Ro15-4513, (3-ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]-benzodiazepine-3-carboxylate; BZ, benzodiazepine; CL218-872, triazolopyridazine, 3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine; L838,417, 7-*tert*-butyl-3-(2,5-difluorophenyl)-6-(2-methyl-2*H*-[1,2,4]triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine; TPA003, 4,2′-difluoro-5′-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-a]pyridine-3-yl]biphenyl-2-carbonitrile; TPA023, 7-(1,1-dimethylethyl)-6-(2-ethyl-2*H*-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorphenyl)-1,2,4-triazolo[4,3-*b*]pyridazine; α5IA, 3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methyloxy]-1,2,4-triazolo[3,4-*a*]phthalazine; SR95531, gabazine, 2-(3′-carboxy-2′-propyl)-3-amino-6-*p*-methoxyphenylpyridazinium bromide); gaboxadol, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP).

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increase in $[Cl^-]_i$ might shift the membrane potential toward the firing threshold, causing rebound excitation of neurons (Marty and Llano, 2005). GABA$_A$-R channels can conduct other anions with variable permeability ratios relative to Cl^-. HCO_3^- flux could be physiologically relevant under certain conditions (Kaila et al., 1997). The importance of bicarbonate varies with tissue and is dependent on carbonic anhydrase activity, including the tissue isozyme expression, as well as anion pumps (Rivera et al., 2005).

B. The Cys-Loop Pentameric Ligand-Gated Ion Channel Superfamily

GABA$_A$-Rs are part of the Cys-loop pentameric ligand-gated ion channel (LGIC) superfamily, including nicotinic acetylcholine receptors (nAChRs) (Corringer et al., 2000; Lukas and Bencherif, 2006), glycine receptors (GlyRs) (Breitinger and Becker, 2002), ionotropic 5-HT receptors (5HT$_3$Rs) (Davies et al., 1999; Thompson and Lummis, 2006), and a Zn^{2+}-activated ion channel (Davies et al., 2003). They differ in structure from two additional LGIC families: the tetrameric glutamate receptors (P2X) (Chen and Wyllie, 2006) and the trimeric purine receptors (Khakh et al., 2001; Khakh and North, 2006). Whereas the nAChR, 5-HT$_3$R, and the Zn^{2+}-activated channels are cation-selective channels and thus excitatory, the GABA$_A$-R and GlyR families are anion-selective channels and, thus, with the exceptions noted above, mediate inhibition. All of the subunit members of the Cys-loop LGIC superfamily show sequence homology on the order of 30% identity but even greater similarity at the level of secondary and tertiary structure. Such receptors are all organized as pentameric membrane-spanning proteins surrounding a central pore that forms the ion channel through the membrane (Fig. 1). They all use similar sequences and functional domains to establish membrane topology, ion channel structure, agonist binding sites, and even binding sites for diverse allosteric ligands. Each subunit consists of a long N-terminal extracellular hydrophilic region, followed by four transmembrane (M) α-helices with a large intracellular loop between M3 and M4, and ends with a relatively short extracellular C-terminal domain. M2 forms the lining of the ion channel, with a possible contribution from M1 (Corringer et al., 2000; Sine and Engel, 2006). An α-helical domain within the M3–M4 cytoplasmic loop has also been shown to influence ion conduction (Peters et al., 2005). The structure of the Cys-loop LGIC superfamily has been investigated by a large number of biochemical approaches, with current work emphasizing domains for ligand binding and coupling to ion channel gating, as well as subunit-subunit interactions and investigation of the role of intracellular domains. All of these data were confirmed and fell into place when combined with X-ray crystallography data on the snail soluble acetylcholine binding protein (Brejc et al., 2001), recently followed by that of the water-soluble portion of the nAChR α1 subunit (Dellisanti et al., 2007). The complete channel structure has been progressively well resolved (currently 4 Å) for the nAChR of *Torpedo marmorata* obtained by cryoelectron microscopy and image reconstruction (Miyazawa et al., 2003, Unwin, 2005).

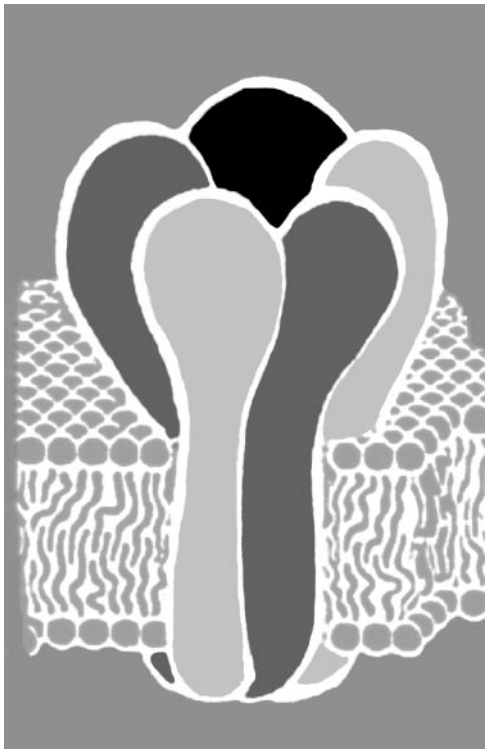

FIG. 1. Schematic rendition of a Cys-loop pentameric ligand-gated ion channel receptor. The different shades of gray signify different subunits (unspecified here), assembled in different permutations to generate multiple hetero-oligomers. The purpose of this review is to attempt to list the naturally occurring subtypes of GABA$_A$ receptors. Similar chores face those interested in other members of the superfamily as well as other structural types of LGIC. [Adapted from Haefely W (1987) Structure and function of the benzodiazepine receptor. *Chimia* **41:**389–396. Copyright © 1987 Swiss Chemical Society. Used with permission.]

C. The GABA$_A$ Receptor Family of 19 Genes

With the complete sequence of the genome for human and a few other vertebrate species, it is now clear that there are 19 genes for GABA$_A$-Rs (Fig. 2) (Simon et al., 2004). These include 16 subunits (α1–6, β1–3, γ1–3, δ, ε, θ, and π) combined as GABA$_A$ and 3 ρ subunits, which contribute to what have sometimes been called GABA$_C$ receptors. Birds and probably some other species additionally express β4 and γ4, but lack θ and ε subunits, so also total 19. The sequence of ε is closely related to γ subunits (Fig. 2) and θ to β, suggesting an evolutionary relationship, perhaps more evident in birds than mammals. The list is modified from Barnard et al. (1998) in that we include the θ, but not the β4, subunit.

D. The ρ Subunits and the GABA$_C$ Receptor Concept: Not Recommended

"GABA$_C$ receptors" were originally described preclon-ing, based on a distinctive pharmacology, to encompass

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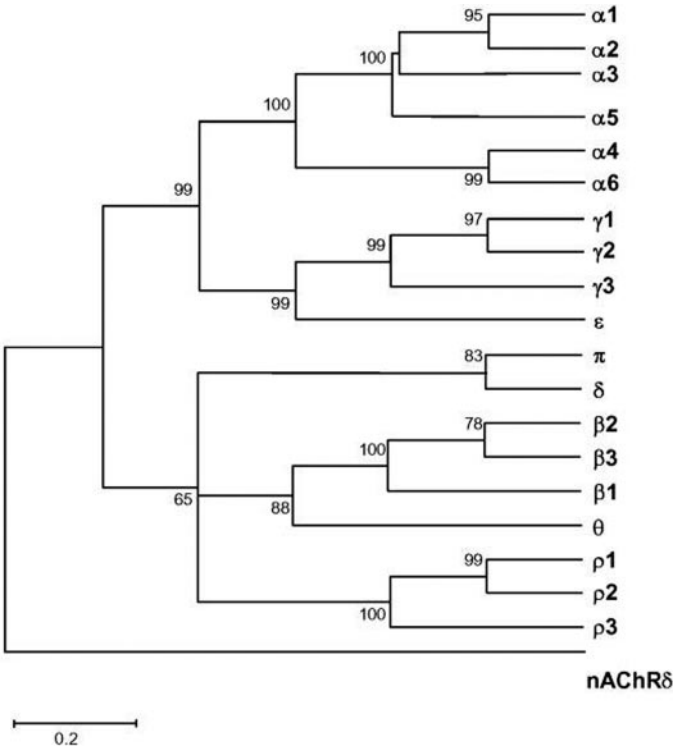

FIG. 2. Dendrogram of the known 19 genes for human $GABA_A$ receptors. The distances along each line are proportional to the degree of sequence identity between the different homologous subunits. The Greek letters signify subunit families of high (>70%) identity, with different Greek letter subunit families showing homology but lower sequence identity. The distances reflect the evolutionary times required to generate sufficient sequence divergence. [Reproduced from Simon J, Wakimoto H, Fujita N, Lalande M, and Barnard EA (2004) Analysis of the set of $GABA_A$ receptor genes in the human genome. *J Biol Chem* **279:**41422–41435. Copyright © 2004. Used with permission.]

responses to GABA that did not fit either the "A" (blocked by bicuculline) or "B" (activated by baclofen) category. Such "nonA, nonB" GABA responses were found in numerous regions of the CNS. Attempts to find such a nonA, nonB GABA-activated current by expressing poly(A)$^+$ RNA from brain in oocytes were unsuccessful but a cDNA expressing this sort of GABA-evoked current was isolated from retina (Woodward et al.,1992). After cloning of the various $GABA_A$ receptor subunit genes, it was demonstrated that the ρ subunits are closely related in sequence, structure, and function to the other $GABA_A$-R subunit families designated with other Greek letters and thus qualify for inclusion within that family. It is now clear that there are three subtype genes of ρ subunits that make homopentameric chloride channels. Such ρ-receptors show some of the pharmacological properties of the $GABA_C$ receptors, such as sensitivity to the GABA analog, CACA (*cis*-aminocrotonic acid). They are relatively insensitive to bicuculline and also to $GABA_A$-R modulators such as benzodiazepines, barbiturates, and general anesthetics, at appropriate concentrations. $GABA_C$ receptors are sensitive to picrotoxin, neurosteroids, and some other drugs, but the overall pharmacology differs from that of most traditional $GABA_A$-Rs (Bormann and Feigenspan, 1995; Johnston, 1996). The ρ subunits are not simply equivalent to $GABA_C$ receptors, because some regions of the nervous system seem to lack ρ subunits and yet exhibit $GABA_C$ (i.e., non-A, non-B) pharmacology. The ρ subunits are all expressed primarily in the retina, but unlike the ρ1, the ρ2 and ρ3 subunits are also found elsewhere (Johnston, 2002). At this time it is not clear whether ρ1–3 can combine with each other in heteromers. Enz and Cutting (1999) claimed that recombinant ρ1 and ρ2 subunits can form hetero-oligomers with distinct physical properties. In native receptors this is difficult to prove because there are no antibodies that distinguish between different ρ subunits. Nor is it established whether ρ1–3 can combine in nature with members of the other 16 $GABA_A$-R subunits. Some evidence suggests these possibilities but it is not decisive (Sieghart and Ernst, 2005). For example, coassembly of ρ and γ2 subunits was reported (Milligan et al., 2004; Pan and Qian 2005), and some cells in hippocampus show $GABA_A$-*R*-like properties intermediate between ρ and γ2-containing $GABA_A$-Rs (Hartmann et al., 2004). There is also some evidence for association of ρ with GlyRs (Pan et al., 2000).

The close structural similarities of ρ subunits to the other $GABA_A$-R subunits, the similarities in anion channel structure and function, the important fact that other subtypes of $GABA_A$-Rs differ in pharmacology, such as benzodiazepine sensitivity, from each other to a similar degree as do the ρ receptors, and the possibility of ρ subunits partnering with other $GABA_A$-R subunits, led to the decision of the Nomenclature Committee of IUPHAR to designate the GABA ρ receptors as part of the $GABA_A$-R family and to recommend against the use of the term $GABA_C$ receptor. It is especially recommended that the name $GABA_C$ receptor should not be used as the sole name for the ρ receptors in an article including, especially, the title and abstract. The ρ subunits are also discussed in section III.C.1.a.

II. Structural Basis of Receptor Classification

A. $GABA_A$ Receptor Family and Superfamily (Homology of Structure and Function)

The sequence homology of all 19 $GABA_A$-R gene products to each other and to the Cys-loop pentameric LGIC superfamily lends itself to attempts to classify the receptors collectively. All members of the superfamily are homologous not only in the domains specifying membrane topology but also at the functional domain level, including ligand binding sites (Sigel and Buhr, 1997; Corringer et al., 2000; Olsen and Sawyer, 2004; Sine and Engel, 2006). Thus, homologous M2 residues are involved in channel structure and ion selectivity (Keramides et al., 2004), and the same multiple loops of extracellular sequence domains contribute to agonist/antagonist binding pockets (Galzi and Changeux, 1994; Sigel, 2002). This allows homology structural modeling

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of the various functional domains comparing GABA$_A$-Rs and nAChRs (Cromer et al., 2002; Ernst et al., 2003; Ernst et al., 2005). Even the binding sites for some allosteric modulators also show considerable homology with agonist binding sites. For example, the major benzodiazepine binding site lies at the α/γ subunit interface and involves residues homologous to the agonist binding loops at the β/α interface, which in turn are homologous in all members of the superfamily (Smith and Olsen, 1995; Sigel and Buhr, 1997; Corringer et al., 2000).

1. Subunit Gene List. The GABA$_A$-R receptor subunit genes form a family of 19 (Table 1).

2. Subunit Splice Variants. Splice variants have been reported for only a few GABA$_A$-R subunits, primarily γ2 (Whiting et al., 1990; Kofuji et al., 1991). The γ2 subunit splice variants differ in only an eight-amino acid stretch of the large intracellular loop that is present in the γ2L and missing in the γ2S subunit. The sequence includes a consensus protein kinase C phosphorylation substrate serine. No functional consequence of phosphorylation of the unique γ2L serine residue has been convincingly demonstrated. The γ2 splice variants are both expressed and show differential abundance in different brain regions (Gutiérrez et al., 1994; Meier and Grantyn, 2004). In addition, they show differential aging-related changes in their level of expression (Gutiérrez et al., 1996). Brain membranes from genetically engineered mice expressing only the γ2S exhibit increased affinity for benzodiazepine agonists, an effect paralleled by increased sensitivity to such compounds in behavioral responses [e.g., increased "sleep" times (loss of righting reflex)] in null allele mice (Quinlan et al., 2000). The subunit composition of the receptors responsible for such changes is unknown, and, indeed, receptors produced in compensation might include normally non-native oligomers.

Alternative splice products in the intracellular loop in β2 and γ3 subunits are found that, like γ2L, also include consensus phosphorylation substrate sequences. Alternative start sites create multiple mRNA species for β3, α3, and α5 subunits, and variants lacking one or more exons have been found for ρ1, α4, β2, and ε subunits (reviewed in Simon et al., 2004). To date, and this could change, none of these variant polypeptides have been demonstrated to be present within functional receptors, nor do they confer any unique function or pharmacology in recombinant expression systems. There is at least one report of RNA editing in the GABA$_A$-R family (Ohlson et al., 2007), but no evidence so far for functional relevance.

TABLE 1
GABA$_A$ receptor subunit gene list
Chromosome data from Simon et al. (2004).

Subunit	Gene	Chromosome		
		Human	Mouse	Rat
α1	*GABRA1*	5q34	11	10
α2	*GABRA2*	4p12	5	14
α3	*GABRA3*	Xq28	X	X
α4	*GABRA4*	4p12	5	14
α5	*GABRA5*	15q13.2	7	1
α6	*GABRA6*	5q34	11	10
β1	*GABRB1*	4p12	5	14
β2	*GABRB2*	5q34	11	10
β3	*GABRB3*	15q13.2	7	1
γ1	*GABRG1*	4p12	5	14
γ2	*GABRG2*	5q34	11	10
γ3	*GABRG3*	15q13.2	7	1
δ	*GARBD*	1p36.3	4	5
ϵ	*GABRE*	Xq28	X	X
θ	*GABRQ*	Xq28	X	X
π	*GABRP*	5q35.1	11	10
ρ1	*GABRR1*	6q15	4	5
ρ2	*GABRR2*	6q15	4	5
ρ3	*GABRR3*	3q12.1	16	11

B. Heteropentameric Assembly Produces Complex Subtype Heterogeneity

The assembly of GABA$_A$-R as heteropentamers produces complex subtype heterogeneity in structure, which is the major determinant of their pharmacological profile. These various subtypes differ in abundance in cells throughout the nervous system and thus in functions related to the circuits involved. A major factor in producing heterogeneity is the existence of the six different α subunit variants. Some of these physiological receptor subtypes containing specific α subunits can be distinguished by ligands that act at the benzodiazepine site (Barnard et al., 1998). In addition, there are now clear examples of β and γ or δ or other subunit selectivity for drug action on GABA$_A$-R subtypes (see section III.B.2). Clearly it is the nature, stoichiometry, and arrangement of the subunits that determine details of pharmacological selectivity. Thus, pharmacology can often provide evidence for structural heterogeneity and informs us about the subunit composition of native receptor subtypes.

Evidence is greatly in favor of a pentameric receptor and most GABA$_A$-R subtypes are formed from two copies of a single α, two copies of a single β, and one copy of another subunit, such as γ, δ, or ε (Sieghart and Sperk, 2002; Olsen and Sawyer, 2004). At this time the subunit composition, stoichiometry, and wheel alignment are not known for most pharmacological subtypes: tentative definitions of subtypes can be provided as a work in progress. Current knowledge allows elimination of most of the thousands of permutations theoretically possible for combinations of the known 19 subunits into five-part (pentameric) complexes. Barnard et al. (1998) suggested a maximum on the order of 800 combinations and probably far fewer in reality on the basis of current knowledge of identified subunit partnering and apparent rules of assembly in neurons. Very few combinations have been conclusively identified in situ. We suggest some criteria for inclusion in a list of native subtypes and begin to generate such a list. Current evidence suggests that only 11 subtypes can be listed as conclusively identified, and these are reasonably abundant. We have also listed several subtypes for which the evidence is strong but not conclusive (six subtypes). We finish by mentioning subtypes containing one of each of the minor sub-

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units, evidence for whose native existence is tentative (another eight, plus one subtype with two kinds of α subunit), for a grand total of 26. An additional similar number of subtypes, which are relatively rare, but are likely to exist, are not listed at this time, but the list will continue to grow as more information becomes available. Each subtype could play a significant role in the cells in which they occur. It should be noted that even these minor $GABA_A$-R subtypes are present in amounts comparable with or greater than receptor subtypes for accepted brain neurotransmitters other than glutamate, that is, the biogenic amines and acetylcholine. The heteromeric LGICs clearly offer much greater heterogeneity than other known receptor subtypes.

C. Criteria for Inclusion on a List of Native Receptor Subtypes

1. Subtypes Based on Structure, Pharmacology, and Function: Nomenclature Guidelines. Criteria are needed to define which receptor subtypes can be accepted as being native to neurons. We suggest basing the criteria on structure, pharmacology, and function. The list of subtypes will necessarily be a work in progress as information on all LGIC families is currently incomplete. We propose five major criteria: two for recombinant studies and three for native studies, each with subclassifications, for inclusion of a subunit combination on the native receptor subtype list (Table 2). None of these criteria by itself is sufficient. Because five criteria are rarely, if ever, met, it is the remit of a committee of experts (e.g., Nomenclature Committee of the IUPHAR subcommittee) to decide which candidates qualify. At this time we choose to be relatively strict regarding inclusion, including subtypes that *either must, or are highly likely* to, exist, rather than including all subtypes that *might* exist. We have decided to include more possibilities in the list for $GABA_A$-Rs (Table 3) by dividing it into three categories, depending on the number of criteria that are met: A) "identified," B) "existence with high probability," and C) "tentative."

2. Discussion of the Criteria. A brief discussion of the available pertinent evidence and its value in determining the existence of native subtypes is in order. The expression of recombinant receptors, the determination of their subunit composition and arrangement, and their biophysical and pharmacological characterization is obviously an important part in the identification of native receptors. But expression of recombinant receptors is insufficient to prove their existence in vivo, because gross overexpression of subunit proteins can result in heterooligomeric combinations that are unlikely to occur in nature. For example, recombinant receptors containing both the $\gamma 2$ and δ subunits have been generated and characterized (Saxena and Macdonald, 1994; Hevers et al., 2000), whereas the actual existence of such receptors in the brain is highly questionable (for discussion, see Sieghart and Sperk, 2002). In addition, coexpression of subunits does not always result in the exclusive formation of the expected receptors. Thus, several reports have shown that coexpression of α, β, and other (γ, δ, and ε) subunits in oocytes or cell lines can lead to considerable expression of $\alpha\beta$ receptor channels, without γ, δ, or ε. One must either precipitate the receptors via the third component subunit measuring subunit composition by Western blots and pharmacological properties via binding studies or use a pharmacological test to demonstrate the expression of this subunit and its contribution to the channel currents. In any case, it has to be kept in mind that receptor heterogeneity might influence results of the subsequent characterization of recombinant receptors. To increase the formation of receptors containing α and β, as well as γ, δ, or ε subunits, higher amounts of mRNA or cDNA for the third component subunit often are used, as well as longer times for expression (Boileau et al., 2002, 2005; Olsen et al., 2007), despite the risk of non-natural or pathological pentamers with too many copies of a given subunit by the use of such extremes.

Thus, in recombinant expression studies, one must demonstrate that the subunits under study are indeed ex-

TABLE 2
Criteria for inclusion on a list of native receptor subtypes

- I. Recombinant receptors
  - A. Evidence for their formation, subunit composition, and stoichiometry, using recombinant receptor expression in heterologous cell systems
    1. The subunit polypeptides must be shown to be expressed
    2. The subunit polypeptides must be shown to coassemble
       - a. Coimmunoprecipitation
       - b. Physical demonstration of subunit interactions by FRET or similar technique
       - c. Formation of pentamers
       - d. Unique subunit arrangement, e.g., using concatemers
    3. The corresponding recombinant receptor subtype must be functional
  - B. Evidence for unique properties, including pharmacology
    1. Unique biophysical characteristics
    2. Unique pharmacology
       - a. Receptor subtype-selective agonists, antagonists, allosteric modulators
       - b. Receptor subtype-selective radioligands
       - c. Potency and efficacy for a series of ligands
       - d. Macrokinetic measures (e.g., apparent EC_{50} values and binding constants for a series of ligands)
- II. Native receptors
  - A. Colocalization of subunits
    1. Tissue colocalization
    2. Cell colocalization (in situ, single-cell RT-PCR)
    3. Subcellular colocalization (light and electron microscopy)
  - B. Physical demonstration of subunit interactions (e.g., by coimmunoprecipitation)
  - C. Functional demonstration:
    1. Evidence that a given receptor is expressed in real neurons by showing properties (assessed with electrophysiology) corresponding with a recombinant receptor candidate; microscopy may complement
    2. Evidence that a given subunit or subunit combination participates in a specific function in vitro or in vivo using genetically modified mice

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pressed, combine into receptors, form pentamers with defined subunit stoichiometry and arrangement, and have function. If possible, the properties of receptors should be compared with those of receptors with defined subunit composition and arrangement using concatemers. One might add that researchers also need to pay attention to the source of their receptor subunit clones and specify the source and sequences of those they use. A single point mutation in the gene of a subunit isolated from the brain could change the properties of the resulting recombinant receptors (Sigel et al., 1992). Then one can examine the receptor for unique biophysical properties using electrophysiology, assessing agonist and modulator mechanisms, determined, for example, from single channel and macroscopic current kinetics, single channel conductance, and possibly conductance substates. Finally, one can examine the receptor for unique pharmacology, using subtype-selective ligands, including radioligands, and measuring relative affinities, potencies, and efficacies for a series of ligands.

Expression in the brain at the mRNA (in situ hybridization, RT-PCR, single cell RT-PCR) or protein level (immunostaining) is a good starting point to indicate that two given subunits may be coexpressed in a given cell and which types of cells express them. A combination of in situ hybridization and immunohistochemistry is stronger than either technique alone. But colocalization of two subunits, although necessary, is not sufficient to establish whether these subunits are partners in a pentameric receptor subtype. Individual subunits alone most often do not define a receptor and might even have functions different from those of receptors. Microscopic colocalization of subunit proteins is one of the factors used in deciding native subtype composition, but colocalization at the light microscope level is not conclusive evidence for coexpression within a pentamer. Likewise, colocalization at the electron microscope level is useful evidence but does not necessarily define receptor subtypes, because even subunits located side-by-side with immunogold labeling could belong to adjacent receptors. Anatomy compendia are being developed by experts, and these play a role in deciding on inclusion of native subtypes. The questionable specificity of many antibodies, however, suggests that great care must be taken to analyze the published work on subunit localization (Rhodes and Trimmer, 2006; Moser et al., 2007). Because most antibodies used are polyclonal and because the composition of polyclonal antibodies is different in each donating animal and even in each blood sample, such verification of the specificity of the antibodies has to go on until highly selective and well characterized monoclonal or recombinant antibodies are available. All antibodies used for these compendia should thus be used in wild-type and knockout mice to be sure that they unequivocally identify the correct protein only. But even if the data are correct, most data on localization are not in themselves sufficient for defining receptor subtype composition.

Coimmunoprecipitation of subunits comes close to defining a subtype because it indicates (but not necessarily proves) assembly. Here again, the antibodies used must be demonstrated to have total subunit specificity as any cross-reactivity contaminates the results. The antibodies must be characterized by Western blotting on crude brain tissue or cells and be shown to recognize the band of correct size, and only that, in normal but not knockout mice, when such mice are available. Specific antibody reagents are currently available for such an approach, and the provider of such antibodies should be asked for data documenting the absence of cross-reactivity. Nevertheless, incompletely assembled intermediates could contribute, and subunits or receptors could associate with each other (natural interaction, artifactual aggregation, or association via cytoskeleton proteins) without being in the same receptors, weakening the strength of the evidence based on the coimmunoprecipitation method. One needs to be careful with the choice of detergent and solubilization conditions to minimize nonspecific protein interactions without destroying oligomers. If one could demonstrate that the associated subunits were present in a detergent-solubilized protein of the correct size for a pentamer this would strengthen the argument, but this is rarely, if ever, provided, and even such studies may be ambiguous because of comigration of multiple heterooligomeric protein species, so the results in any case must be regarded with caution. In the case of G protein-coupled receptor heteromultimer possibilities, evidence for true subunit-subunit association considers physical techniques such as FRET (Pin et al., 2007), and this approach may also find use for ligand-gated ion channel receptors. However, the method is extremely difficult to apply to native proteins, requiring the use of transgenic mice. If it is performed with recombinant receptors in a heterologous expression system, there is again the possibility of identifying non-native combinations owing to subunit overexpression. But such studies again would not provide a clear-cut answer because subunits demonstrated to be close together by FRET techniques could have assembled in the same receptor or be located in two different receptors associated with each other. The above considerations seem trivial, but they are not, judged by proposals of acknowledged scientists on how to unequivocally solve the problems of establishing receptor subunit composition that do not survive more thorough discussions.

Thus, the conclusion is that a combination of different techniques has to be used to identify a receptor subunit composition of a native receptor, knowing the limits of each technique. Even then, one cannot be absolutely sure, but at least this is at the limit of the techniques available. What is really needed is "in situ" electrophysiology with a channel characterization and a pharmacological fingerprint of the receptors to prove that the

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subunits known to exist in that cell contribute to the receptor studied and its physiology. This is extremely difficult, and fingerprints for the different receptor subtypes are not always sufficiently selective, may be host cell-specific, or are lacking altogether. One must take care to be as certain as possible that the subunits meant to be expressed in a recombinant system for obtaining a fingerprint are actually present and contributing to the data and that the pentamer has correct stoichiometry (see discussion above). Recombinant expression suffers from uncertainty that perhaps other gene products present in neurons might not be present in the cells used for recombinant expression (e.g., associated proteins, proteins affecting trafficking, or post-translational modifications) or that they do not exactly match that in the native system because of host cell-specific factors (Birnir and Korpi, 2007). Even a biophysical characteristic as fundamental as single-channel conductance can be affected by the expression system (Lewis et al., 1997), and anecdotes abound regarding different results for the "same receptors" expressed in two kinds of cells. Concatenated subunits may be of use in determining specific subunit composition and wheel arrangement (Im et al., 1995; Baumann et al., 2002; Minier and Sigel, 2004; Boileau et al., 2005; Sigel et al., 2006), although this approach also has a number of problems (Ericksen and Boileau, 2007).

To summarize the strategy used: one first notes which subunits are expressed in a given cell type. Then one looks at evidence that a given pair or triplet of subunits on that list are associated, based on coimmunoprecipitation, coregulation in cells, or knockout animals. The coexpression of $\alpha1\beta2\gamma2$ suggests the possibility of coordinate regulation of expression of chromosomal clusters, but the exceptions are too abundant to consider this a factor in partnering. Then one determines by recombinant expression and electrophysiology the channel kinetics and pharmacology of those subunit combinations, keeping in mind the numerous caveats listed above. Because most of these caveats have not been taken into account previously, one clearly cannot believe everything published on the properties of recombinant receptors. Native receptors with the same unique properties are then sought in the neurons recorded from brain slices, dissociated single cells, or at least cells in culture.

Genetically engineered mice (knockout and knockin) provide some evidence indicating that certain oligomeric receptors really do exist because one can correlate the loss of certain receptor responses and behaviors with the receptor subtypes addressed (Jones et al., 1997; Rudolph et al., 1999; McKernan et al., 2000; Sur et al., 2001; Vicini et al., 2001). The function of deleted genes is sometimes obvious, whereas in other cases, subtle changes in behavior need to be evaluated properly. Evidence from global knockout mice is often complicated by possible compensation, causing the observer to miss important functions. For example, deletion of the $\alpha1$ subunit of the $GABA_A$-R results in loss of nearly 50% of the total $GABA_A$-R population in mouse brain and ablates fast synaptic transmission mediated by the abundant $\alpha1$ subunit-containing receptors, yet the null mutant mice display a phenotype that is grossly normal (Sur et al., 2001). Diverse transcriptional responses may act to abrogate the effect of the knockout and preserve neuronal excitability and network behavior (Ponomarev et al., 2006). Point mutated knockin mice with altered behavior/pharmacology are more convincing, and several important examples are included in the $GABA_A$-R field. For an overview and discussion, see Olsen and Homanics (2000), Rudolph and Möhler (2004), Sieghart and Ernst (2005), and Atack (2005).

III. Working List of Native $GABA_A$ Receptor Subtypes

A. Evidence for Subtypes from Localization, Abundance, Subunit Composition, and Stoichiometry Data

It was found early on in recombinant $GABA_A$-R studies that robust GABA-activated channel formation occurred with combinations of α and β subunits and also with α, β, and γ subunits. The latter turned out to be the prevalent native combination (Barnard et al., 1998). The vast majority of $GABA_A$-Rs in the CNS contain the $\gamma2$ subunit, and this is the most abundant subunit in rat brain and in most regions based on in situ hybridization of mRNA (Wisden et al., 1992; Laurie et al., 1992; Persohn et al., 1991, 1992) and immunostaining (Fritschy and Möhler, 1995; Pirker et al., 2000). The $\gamma1$ and $\gamma3$ subunits are rarer but have some role in discrete regions, probably with well prescribed subunit partners. Thus, approximately 75 to 80% of $GABA_A$-Rs contain the $\gamma2$ subunit (Whiting et al., 2000; Sieghart and Ernst, 2005).

The $\alpha1$ is the most abundant of the α subunits and is often colocalized with its chromosome partners, the likewise highly expressed $\beta2$ and $\gamma2$ subunits (Sieghart and Sperk, 2002). As noted above, knockout of the $\alpha1$ subunit causes total $GABA_A$-R content in mouse brain to decrease by 50% (Sur et al., 2001). The $\alpha2$ and $\alpha3$ subunits are moderately abundant and $\alpha5$ is relatively rare except in the hippocampus, as indicated by regional distribution and immunoprecipitation studies (Pirker et al., 2000; Sieghart and Sperk, 2002). The $\alpha4$ and $\alpha6$ subunits are reasonably highly expressed in forebrain and cerebellum, respectively. Among the β subunits, $\beta1$ is least common, $\beta2$ is most abundant and most widespread (knockout results in a 50% reduction in $GABA_A$-Rs in mouse brain) (Sur et al., 2001), and $\beta3$ is reasonably highly expressed, but more discrete. Furthermore, it is more dense perinatally than in adult brain (Zhang et al., 1991; Laurie et al., 1992). The identity of the β subunit often cannot be determined, because on precipitation of $GABA_A$-Rs with α, γ, or δ subunit-specific antibodies in

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most cases all three β subunits are coprecipitated. Only in some rare areas where there are cells with only one type of β subunit contributing to functional GABA$_A$-Rs (Persohn et al., 1991, 1992; Wisden et al., 1992; Laurie et al., 1992; Pirker et al., 2000) can the type of β subunit be predicted. In these areas, however, no subsequent immunoprecipitation or electrophysiological studies have been performed to investigate possible assembly partners, and, thus, actual assembly of defined receptors and their composition cannot be derived with certainty. For this reason, it has been concluded that all of the β subunits exist in functional receptors, usually with only one type per pentamer (Whiting et al., 2000).

Based on the frequent colocalization in the brain (Fritschy and Möhler, 1995; Pirker et al., 2000) and in neurons (Klausberger et al., 2002), on results from coimmunoprecipitation experiments (Jechlinger et al., 1998; Pöltl et al., 2003), and on the pharmacology described in section III.B, the $\alpha1\beta2\gamma2$ subunit combination is considered to exist in the brain, probably in large amounts (Benke et al., 1991; Somogyi et al., 1996; Whiting et al., 2000). Recombinant $\alpha1\beta2\gamma2$ receptors have been shown to have a 2α-2β-$1\gamma2$ stoichiometry [denoted $(\alpha1)_2(\beta2)_2\gamma2$], and this stoichiometry is supported by coimmunoprecipitation results from rat or mouse brain (Sieghart and Sperk, 2002). It is generally assumed, without conclusive proof, that other α and β subunits also combine with $\gamma2$ in combinations of 2α-2β-$1\gamma2$ (Sieghart and Ernst, 2005). Other α subunits are also colocalized with and have been shown to coprecipitate with all β and the $\gamma2$ subunits (Sieghart and Sperk, 2002). Receptors containing these subunits also have a specific pharmacology (see section III.B) and thus also seem to exist in the brain.

The other "minor" subunits, notably δ, are thought to be able to replace γ in the pentamer, as in the 2α-2β-δ combination (Barrera et al., 2008). However, $\alpha4$ and $\alpha6$ subunits are just as often combined with the δ subunit as with $\gamma2$ (Sieghart and Sperk, 2002). The δ subunit is obligatorily partnered with the $\alpha6$ subunit in cerebellar granule cells and is primarily associated with the $\alpha4$ subunit in forebrain areas including dentate gyrus, neostriatum, some layers of cortex, and a few other areas. The δ subunit seems to have a perisynaptic/extrasynaptic localization (Nusser et al., 1998; Peng et al., 2002; Wei et al., 2003). The ε subunit is rare but can substitute for γ or δ in some areas, such as hypothalamus, and the θ and π subunits are only sketchily characterized (Korpi et al., 2002; Sieghart and Sperk, 2002).

The $\gamma2$ subunit is required for synaptic localization of GABA$_A$-Rs, usually associated with the $\alpha1$, $\alpha2$, or $\alpha3$ subunits. Nevertheless, substantial numbers, possibly a majority, of $\gamma2$ subunit-containing GABA$_A$-Rs are extrasynaptic, because of the much greater area of extrasynaptic membranes. This includes some of the $\alpha1/\alpha2/\alpha3$ and the great majority of $\alpha4/\alpha5/\alpha6$ combinations. In contrast, combinations in which other subunits, such as δ and ε, replace $\gamma2$ are considered to be exclusively nonsynaptic. The physiological and pharmacological importance of perisynaptically and extrasynaptically localized GABA$_A$-Rs has recently become increasingly appreciated (Mody and Pearce, 2004; Semyanov et al., 2004; Farrant and Nusser, 2005).

B. Pharmacological Evidence for Subtypes

1. Benzodiazepine Site Ligands Distinguish between Subtypes Based on α and γ Subunits. The typical GABA$_A$-R is positively modulated by diazepam-like benzodiazepines and binds radioligands for the GABA site (e.g., [^{3}H]muscimol), the benzodiazepine site (e.g., [^{3}H]flunitrazepam, [^{3}H]flumazenil, or [^{3}H]Ro15-4513, and the picrotoxin/convulsant/ channel sites (e.g., [^{35}S]*t*-butyl bicyclophosphorothionate) (reviewed by Macdonald and Olsen, 1994; Sieghart, 1995; Barnard et al., 1998; Korpi et al., 2002; Johnston, 2005). Barnard et al. (1998) listed approximately 20 chemical classes of ligand that bind to the benzodiazepine (BZ) site on GABA$_A$-R, including the structures of approximately 40 compounds. Additional chemical classes of ligands are listed in Gardner et al. (1993), Olsen and Gordey (2000), Sieghart and Ernst (2005), Atack (2005), and Whiting (2006), which should be consulted for chemical names and structures. In each of these chemical classes compounds are available that enhance (positive allosteric modulators or BZ site agonists) or reduce (negative allosteric modulators or inverse BZ site agonists) GABA-induced chloride ion flux via the same BZ binding site. In addition, in each of these chemical classes there are compounds that do not modulate GABA-induced chloride flux, although they interact with the BZ binding site (neutral BZ site ligands or BZ site antagonists). The latter compounds, however, are able to inhibit the action of BZ site agonists or inverse agonists. In between the full BZ site agonists or inverse agonists, there are compounds that elicit less drastic allosteric enhancement or reduction of GABA-induced chloride currents; these compounds are called partial agonists or partial inverse agonists at the BZ site, respectively.

The BZ binding site is located at the interface of an α and a γ subunit. Its pharmacology is thus influenced by both of these subunits, whereas β subunits, although needed to construct a channel, do not greatly affect the sensitivity of the GABA$_A$-R to BZ site ligands (Hadingham et al., 1993). The traditional BZ site agonists (GABA-enhancing CNS depressants such as diazepam) are active on the GABA$_A$-Rs containing a $\gamma2$ subunit (Pritchett et al., 1989), a β subunit, and one of the α subunits, α1, 2, 3, or 5. Receptors containing the $\gamma2$ subunit exhibit a higher BZ sensitivity than those containing the $\gamma1$ subunit (Sieghart, 1995; Khom et al., 2006); the $\gamma3$-containing GABA$_A$-Rs are modulated by some BZ ligands but with altered selectivity from those incorporating the $\gamma2$ subunit (Sieghart, 1995; Hevers and Lüddens, 1998).The BZ-sensitive GABA$_A$-Rs can

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be further subdivided, in that receptors containing the α1 subunit have a higher sensitivity to a subpopulation of BZ site ligands, the benzodiazepines quazepam and cinolazepam (Sieghart, 1989) or nonbenzodiazepines such as zolpidem (an imidazopyridine) and a few others, including CL218-872 (triazolopyridazine), zaleplon, and indiplon, and abecarnil (β-carboline), (Olsen and Gordey, 2000; Korpi et al., 2002; Sieghart and Ernst, 2005). Furthermore, receptors containing the α2 or α3 subunit have an intermediate affinity for zolpidem, whereas those containing α5 have very low affinity for this drug. The differential zolpidem affinity demonstrated by recombinant $GABA_A$-Rs containing different α subunits can also be found in the brain (Itier et al., 1996; Whiting et al., 2000; Sieghart and Sperk, 2002) and individual cells can be shown to exhibit more than one $GABA_A$-R with varying affinity for zolpidem, depending on α subunit subtype expression (Criswell et al., 1997). Again, consult the cited reviews for chemical structures and analogs.

Receptors containing the α4 or α6 subunits, together with β and γ2, do not bind the traditional BZ agonists, including zolpidem, but demonstrate high affinity for some ligands, notably the imidazobenzodiazepines such as flumazenil and Ro15-4513, or bretazenil (Korpi et al., 2002). Both the potency and efficacy for BZ ligands depend on the nature of the α subunit.

The benzodiazepine site ligands so far available do not distinguish well between the α2 and α3 or between the α4 and α6 subunits. All four, however, can produce functional channels in vitro when coexpressed with other subunits, and their differential distributions in the brain suggest they will modulate different behavioral circuitry. Behavioral effects of drugs predicted from subtype selectivity in vitro has been successful in a few cases, such as the α2/3-selective triazolopyridazine anxiolytics based on CL218-872: L838,417, TPA003, or TPA023 (Atack, 2005; Morris et al., 2006; Atack et al., 2006).

Subtype selectivity for drugs in vivo has been further confirmed in subunit-specific genetically engineered mice (Rudolph and Möhler, 2004; Atack, 2005; Whiting, 2006). Thus, based on the evidence that most of the actions of diazepam are mediated via receptors composed of $\alpha1\beta\gamma2$, $\alpha2\beta\gamma2$, $\alpha3\beta\gamma2$, and $\alpha5\beta\gamma2$ subunits, a point mutation involving a histidine to arginine substitution was introduced into the genes of the individual α subunits, rendering the respective receptors insensitive to allosteric modulation by diazepam. A comparison of drug-induced behavioral responses in the mutated and wild-type mice then allowed the identification of diazepam effects that were missing, or reduced, in the mutant mice. With this approach, it was demonstrated that $\alpha1\beta\gamma2$ receptors mediate the sedative, anterograde amnestic and in part the anticonvulsant actions of diazepam (Rudolph et al., 1999; McKernan et al., 2000). These two independent studies showed exactly the same behavioral responses, provided the mice were tested under the same conditions (Crestani et al., 2000). The anxiolytic activity of diazepam is mediated by $GABA_A$-Rs composed of $\alpha2\beta\gamma2$ subunits (Low et al., 2000), and, under conditions of high receptor occupancy, also by α3 $GABA_A$-Rs (Dias et al., 2005; Yee et al., 2005; discussed by Möhler, 2007). The α3-selective drug TP003 also implicates a role for $\alpha3\beta\gamma2$ receptors in anxiolytic (Dias et al., 2005) and anticonvulsant action (Fradley et al., 2007). The $\alpha2\beta\gamma2$ and $\alpha3\beta\gamma2$ receptors are also implicated in some of the muscle relaxant activities of diazepam (Low et al., 2000) and the $\alpha3\beta\gamma2$ in the antiabsence effects of clonazepam (Sohal et al., 2003). The α3 global knockout mice displayed a hyperdopaminergic phenotype relevant for GABAergic control of psychotic-like symptoms (Yee et al., 2005). The $\alpha5\beta\gamma2$ receptors seem to influence learning and memory, shown by improved spatial memory in mice with knockout of α5 subunits (Collinson et al., 2000), and trace fear conditioning was facilitated in the point-mutated α5 knockin which unexplainedly showed major α5 subunit knockdown in the CA1 region (Crestani et al., 2002; Rudolph and Möhler, 2004). Furthermore, the α5-selective inverse agonists such as α5IA can improve cognitive function (Atack, 2005; Dawson et al., 2006). Mice lacking the α5 subunit were shown to exhibit reduced amnestic response to the intravenous general anesthetic etomidate but not to the immobilizing activity of the drug (Cheng et al., 2006). Knockout mice have also implicated several $GABA_A$-R subtypes in the reinforcing effects of ethanol, including the α1 (Boehm et al., 2004), the α5 (Boehm et al., 2004; Stephens et al., 2006), the δ (Mihalek et al.,2001), the α4 (Liang et al., 2008), and the α6 subunit (Hanchar et al., 2005). Years of study on α subunit-dependent subtypes have led to drug candidates for modifying selective behaviors and clinical indications (Atack, 2005; Sieghart and Ernst, 2005; Whiting, 2006), and the genetically modified mice have supported this subtype selectivity. Possibly even more impressive than the drug candidates has been the clues generated regarding behavior involving specific receptor subtypes and thus specific brain circuitries (for review, see Rudolph and Möhler, 2004). Although this attribution of the varied behavioral actions of diazepam site ligands and ethanol to different receptor subtypes might only be a first approximation, and the evaluation may be somewhat tentative, it clearly indicates that receptors containing the respective subunits are present in the brain and exhibit distinct functions in different neuronal circuits, verified by consistent results with subtype-selective drugs.

In contrast to $\alpha1\beta\gamma2$, $\alpha2\beta\gamma2$, $\alpha3\beta\gamma2$, and $\alpha5\beta\gamma2$ receptors, $\alpha4\beta\gamma2$ and $\alpha6\beta\gamma2$ receptors are diazepam-insensitive but are still able to bind the imidazobenzodiazepines Ro15-4513, and flumazenil, which act as BZ site inverse agonist and antagonist, respectively. Thus, these receptors can be identified by ligand-binding studies and autoradiography, using [^{3}H]Ro15–4513 in the

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absence or presence of high concentrations of diazepam or flumazenil, to mask receptors containing the α1, α2, α3, or α5 subunits. Such studies have clearly identified diazepam-insensitive, but flumazenil-sensitive, binding sites in cerebellum and forebrain. These sites disappear in α6 or α4 null mutant mice, indicating that they represent α6$\beta\gamma$2, or α4$\beta\gamma$2 receptors, respectively (Korpi et al., 2002). In addition, there are GABA$_A$R-mediated inhibitory currents recorded in hippocampus that are enhanced by Ro15–4513, an effect specific for α4$\beta\gamma$2 (Liang et al., 2006). So, even with limited pharmacology, some in vivo and in vitro information about selectivity, and the help of genetically modified mice, we can make receptor assignments; e.g., each α subunit defines at least one unique subtype, partnered with a β and a γ2 subunit. Thus, GABA$_A$-Rs exist with binding properties consistent with high, intermediate, and low affinity for certain BZ site ligands, such as zolpidem, and GABA$_A$-R channels with this pharmacological specificity are localized in certain brain regions where the different subunits are expressed. In addition, all α subunits can be coprecipitated with β and γ2 subunits in these regions. Therefore, we conclude that the six different α subunits occur in the brain, combined with β and γ2, and can start our receptor list with all six α subunits, each combined with $\beta\gamma$2, for a total of six subtypes (Macdonald and Olsen, 1994; Sieghart, 1995; Barnard et al., 1998; Whiting et al., 2000; Korpi et al., 2002). As discussed in section III.A, only for the α1$\beta\gamma$2 receptor subtype does sufficient evidence identify the type of associated β subunit (α1β2γ2).

The δ subunit, generally partnered with the α4 and α6 subunits, produces the seventh and eighth subtypes. This is supported by evidence of colocalization and coimmunoprecipitation (Jechlinger et al., 1998; Whiting et al., 2000; Pöltl et al., 2003; Sieghart and Ernst, 2005), accompanied by electrophysiological evidence for α6$\beta\delta$ GABA$_A$-Rs in cerebellar granule cells and α4$\beta\delta$ in forebrain, based on tonic BZ-insensitive GABA$_A$-R currents, which are lost in the δ knockout mice (Stell et al., 2003; Wei et al., 2003; Belelli et al., 2005; Hanchar et al., 2005; Liang et al., 2006; Glykys et al., 2007). Such GABA$_A$-Rs bind even fewer BZ site ligands. Nevertheless, these subtypes have recently been shown to bind Ro15–4513, flumazenil, and certain β-carbolines (Hanchar et al., 2006; Wallner et al., 2006). Thus, current evidence supports the existence of α4$\beta\delta$ and α6$\beta\delta$ receptors, and we conclude that these eight GABA$_A$-Rs should be placed in category A as conclusively identified.

2. Evidence from Other Ligands (GABA, Picrotoxin, General Anesthetics, and Ethanol) Confirms and Extends the List of Receptors. Heterogeneity of GABA$_A$-Rs was evident even before the cloning of the individual subunits as indicated by brain regional variation in radioligand binding, inhibition, and allosteric modulation involving the GABA, BZ, convulsant, and anesthetic sites as well as by photolabeling of GABA$_A$-R subunits using radiolabeled benzodiazepines (e.g., Sieghart, 1989; Olsen et al., 1990; Sieghart, 1995). Such evidence for subtypes was consistent with differential pharmacology assessed by electrophysiological recordings conducted using different cells (Macdonald and Olsen, 1994; Hevers and Lüddens, 1998). The potencies of GABA and antagonists such as gabazine (SR95531) vary slightly with the α subunit (Bai et al., 2001), being more potent at α4, 5, and 6, than at α1, 2, and 3 subunit-containing receptors and with the α4$\beta\delta$ and α6$\beta\delta$ receptors showing even higher potency for GABA than the corresponding γ2 subunit-containing GABA$_A$-R (Wallner et al., 2003). The α5$\beta\gamma$2, α4$\beta\delta$, and α6$\beta\delta$ subtypes have recently been shown to be localized in extrasynaptic membranes, where they mediate a tonic inhibitory current, now recognized to have significant physiological relevance in controlling neuronal excitability, relevant to local circuitry and network activity (Bai et al., 2001; Caraiscos et al., 2004; Mody and Pearce, 2004; Farrant and Nusser, 2005). Many of these extrasynaptic GABA$_A$-R show a high affinity for GABA and are resistant to desensitization, appropriate for their role in tonic inhibition. In addition, GABA has a low efficacy for these receptors, making them susceptible to higher efficacy for modulation by allosteric drugs such as nonbenzodiazepines and especially general anesthetics (Brown et al., 2002; Wohlfarth et al., 2002; Stell et al., 2003; Wallner et al., 2003; Caraiscos et al., 2004; Belelli et al., 2005). Overall, these drugs produce a larger charge transfer via extrasynaptic than synaptic receptors, and, in contrast to earlier dogma, the actions of some important drugs seem to involve modulation of extrasynaptic rather than synaptic receptors. Extrasynaptic GABA$_A$-Rs show great heterogeneity with respect to subunit composition and include a significant fraction of the γ2-containing GABA$_A$-Rs, whereas those lacking γ2 are exclusively extrasynaptic.

The synaptic γ2 subunit-containing GABA$_A$-Rs are all modulated by general anesthetics of diverse chemical structure including pentobarbital, etomidate, and propofol, as well as the neuroactive steroids, but the extrasynaptic δ subunit-containing GABA$_A$-Rs, and possibly the α5$\beta\gamma$2, are more sensitive to these drugs (Brown et al., 2002; Wohlfarth et al., 2002; Stell et al., 2003; Wallner et al., 2003; Hemmings et al., 2005; Herd et al., 2007). As mentioned, the extrasynaptic δ subunit-containing α4$\beta\delta$ and α6$\beta\delta$ GABA$_A$-Rs show a significant modulation by nonbenzodiazepine drugs. Recordings in native tissues suggest therefore that both γ2- and δ-containing GABA$_A$-Rs are clearly present and physiologically relevant. Glykys et al. (2007) demonstrated that the δ subunit can pair with α1β in hippocampal interneurons, based on colocalization with immunostaining, electropharmacological properties, and changes in knockout mice. Although this single report is convincing, we decided that this subtype did not meet sufficient criteria to be included in the list of identified subtypes. Rather than omit it, the α1$\beta\delta$ subtype is placed within a second category of native receptors

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(category B, existence with high probability), so that readers are informed of receptors being considered for inclusion and can evaluate for themselves if they believe one qualifies.

The $\gamma 2$ and δ subunits may have selective β partners, but considerable evidence from colocalization, coimmunoprecipitation, and neuronal electrophysiology studies suggests that each partners with more than one β subunit (one at a time), so this provides a framework for additional subtypes. It was shown earlier that the nature of the α subunit might affect modulator dose-response curves, but no effect was found for varying the β subunit (e.g., Hadingham et al., 1993; Thompson et al., 1996; Smith et al., 2004). Nevertheless, mutation of residues in the channel M2 domain *of any subunit*, but definitely showing some subunit selectivity in the details, can change modulator affinity or efficacy (Tierney et al., 1996; Hill-Venning et al., 1997; Thompson et al., 1999). Thus, mutations of residues in the M2 domain of β subunits can produce β subunit selectivity for pharmacological agents (Cestari et al., 1996; Rudolph and Antkowiak, 2004).

In addition, recent evidence suggests that the β subunit can affect both the pharmacological and channel properties of the $GABA_A$-R, especially in the case of certain nonbenzodiazepine modulators and particularly for the extrasynaptic δ subunit-containing receptors. Thus, several modulatory ligands, such as the nonbenzodiazepine loreclezole and chemically related ligands, such as the general anesthetic etomidate, show some selectivity for $\beta 2$ and $\beta 3$ over $\beta 1$ subunit-containing receptors (Wingrove et al., 1994; Hill-Venning et al., 1997): these authors identified a single amino acid residue 15′ in the M2 domain (counting from the intracellular N-terminal end of the channel-forming second transmembrane helix) responsible for this β subunit selectivity. Mutations of this residue were shown to affect sensitivity to the actions of etomidate in vitro (Belelli et al., 1997) and in vivo (Reynolds et al., 2003). A point mutation of the $\beta 2$ subunit N265S that eliminates etomidate sensitivity in vitro loses the sedative-hypnotic effects of etomidate in the mouse knockin (Reynolds et al., 2003), whereas the $\beta 3$ knockin N265M eliminates the immobilizing effect to a noxious stimulus of etomidate and suppresses loss of the righting reflex to this agent, typical of general anesthetics (Jurd et al., 2003).

The knockin mice showing either $\beta 3$ or $\beta 2$ insensitivity to etomidate in vivo allow detective work on which β subunit mediates the action of this drug on a given cell and current, and, along with other information about the physiology and subunit expression pattern in that cell, a good guess at the subunit composition of the functional receptors (e.g., which α subunits go with which β subunit) (Rudolph and Antkowiak, 2004; Belelli et al., 2005; Herd et al., 2008). So far, however, the results did not provide evidence on whether the $\gamma 2$- or δ-containing receptors, or both, mediate the etomidate effects identified, although the δ-containing receptors are more sensitive to modulation.

This residue 15′ in the M2 "channel domain" of β subunits that affects etomidate sensitivity also affects sensitivity and to some degree β subunit selectivity of $GABA_A$-R to other anesthetic modulators such as propofol, barbiturates, and volatile agents (Cestari et al., 1996; Rudolph and Antkowiak, 2004), consistent with the earlier observation that this residue in (presumably all) α and β subunits affects sensitivity to volatile anesthetics and long-chain alcohols and may be part of a binding pocket for these drugs (Mihic et al., 1997; Ernst et al., 2005).

A few other ligands reported to show β selectivity include valerenic acid (Khom et al., 2007), some novel plant substances of a polyacetylene structure (Baur et al., 2005), tracazolate, and mefenamic acid (Korpi et al., 2002; Smith et al., 2004). This may be extended to ethanol (Wallner et al., 2003). The δ subunit-containing $GABA_A$-Rs, partnered with the $\alpha 4$ or $\alpha 6$ subunits and especially with $\beta 3$, are more sensitive than $\gamma 2$-containing receptors to general anesthetics, neurosteroids, GABA analogs such as gaboxadol (Brown et al., 2002; Wohlfarth et al., 2002; Chandra et al., 2006), and taurine (Hadley and Amin, 2007; Jia et al., 2008) as well as ethanol (Wallner et al., 2003; Hanchar et al., 2005). The $\alpha 4\beta 2\delta$ and $\alpha 4\beta 3\delta$ receptors differ in sensitivity to modulators when recombinantly expressed in cells, and both clearly occur naturally, because some brain areas express $\alpha 4$ and δ but only $\beta 2$ or $\beta 3$ subunits and display a pharmacology that distinguishes between these receptors. Thus, thalamic relay nuclei mainly express the $\beta 2$ subunit and the moderately ethanol-sensitive $\alpha 4\beta 2\delta$ receptor definitely mediates the tonic current (Chandra et al., 2006). Dentate granule cells express high levels of highly ethanol-sensitive, presumably $\alpha 4\beta 3\delta$ isoforms (Liang et al., 2006) but additionally express the etomidate-sensitive $\alpha 4\beta 2\delta$ isoforms (Herd et al., 2008). This modulator selectivity for different β subunits in vitro and in vivo is supported by knockin mouse data. Partnering of either $\beta 2$ or $\beta 3$ with both the $\alpha 4\delta$ and $\alpha 6\delta$ subunits thus seems highly likely. To account for this additional evidence indicating the existence of $\alpha 4\delta$ and $\alpha 6\delta$ subunits combined with specific β subunits, we add $\alpha 4\beta 2\delta$ and $\alpha 4\beta 3\delta$, $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ subtypes in category A, replacing the generic $\alpha 4\beta\delta$ and $\alpha 6\beta\delta$ and bringing our total to 10. Subtypes dividing the $\alpha\gamma 2$ subtypes depending on the $\beta 2$ versus $\beta 3$ partner, except for $\alpha 1\beta 2\gamma 2$, will not be listed until some compelling evidence is presented that the variants exist and differ in some property.

One strong candidate that nearly qualifies for category A is the $\alpha 5\beta 3\gamma 2$ subtype. The $\alpha 5$ and $\beta 3$ subunits seem to be codepleted in mice lacking either the $\beta 3$ or $\alpha 5$ subunit (Olsen and Homanics, 2000), and the properties of recombinant $\alpha 5\beta 3\gamma 2$ receptors seem to reflect those of a native subtype found in CA1 pyramidal neurons, cells enriched in both $\alpha 5$ and $\beta 3$ subunits, and distinct from

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receptors in dentate gyrus granule cells (Burgard et al., 1996; Sur et al., 1998; McClennan and Twyman, 1999; Stell et al., 2003; Caraiscos et al., 2004). However, the specific partnering of α5 with β3 has not been shown by coimmunoprecipitation or pharmacological changes in mutant mice, so we place this subtype in category B. Another example in this category is the $\alpha1\beta3\gamma2$ subtype. If we specify that β2 is the most common partner of $\alpha1\gamma2$ and list it in category A, we need to ask whether α1 can partner with other β subunits. Although evidence for β1 is lacking, the properties of $\alpha1\beta3\gamma2$ receptors in recombinant studies resemble those in certain neurons that express these subunits, some of which lack the β2 subunit (Whiting et al., 2000; Sieghart and Sperk, 2002). By contrast, the β partner with $\alpha2\gamma2$ receptors is unknown; again the $\alpha2\beta3\gamma2$ is a likely possibility.

Although the β1 subunit is less abundant than the other β subunits, it is likely that some β1-containing GABA$_A$-Rs exist, based on regional distribution and coimmunoprecipitation data (Li and De Blas, 1997; Jechlinger et al., 1998). The preponderance of the evidence suggests that β1-containing receptors exist. A potential pharmacological fingerprint, salicylidene salicylhydrazide, has been reported to be a negative allosteric modulator selective for β1 versus β2 or β3 subunit-containing receptors (Thompson et al., 2004). There is some evidence (Whiting et al., 2000) that cultured astrocytes express GABA$_A$-Rs with $\alpha2\beta1\gamma1$ combinations, and they are uniquely enhanced by the BZ site ligand methy-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), a β-carboline. More evidence is needed to establish whether this subtype actually exists in brain. The β1 subunit probably partners with both γ or δ subunits, but there is no conclusive evidence for either. At this time we will add just one subtype for $\alpha\beta1\gamma/\alpha\beta1\delta$ in category B.

C. Other, Nonligand, Delineation of Heterogeneity

1. Subunit Composition.

a. Minor subunits (ρ1–3, γ1, γ3, ε, θ, and π). First, evidence from all criteria supports the existence of at least one ρ-containing native receptor so we added ρ to the list in category A (Table 3), for a total of 11 identified receptors. That total is all that we find to meet sufficient criteria for this designation in 2008. However, each of the ρ subunit genes ρ1, ρ2, and ρ3 probably encode at least one unique pharmacological subtype because their products have a differential brain distribution (Enz and Cutting, 1999; Greka et al., 2000). On the other hand, evidence for their separate formation of GABA-activated ion channels is scarce, because of the lack of antibodies for selective immunoprecipitation and the paucity of pharmacological tools suitable for their unequivocal identification as unique receptors. Hence, we place these three in a third category: C, tentative. However, we feel that the possible heteromerization of ρ, plus the possible combination with other GABA$_A$-R or glycine receptor subunits (Pan et al., 2000; Hartmann et al., 2004; Milligan et al., 2004), does not qualify as subtypes for the list at this time.

The evidence for the existence of a native subtype containing any of the five minor subunits (γ1, γ3, ε, θ, and π) is not as compelling as that for the other, more abundant, subunits (α1–6, β1–3, γ2, and δ) already described. This is attributable to a scarcity of studies and the lack of pharmacological tools and in vivo investigations. However, these subunits belong to the GABA$_A$-R family and can form recombinant GABA-activated chloride channels on coexpression with other subunits, (Sieghart, 1995; Hevers and Lüddens, 1998) and exhibit differential regional distribution in the brain (Laurie et al., 1992; Persohn et al., 1992; Wisden et al., 1992; Davies et al., 1997; Hedblom and Kirkness, 1997; Whiting et al., 1997; Bonnert et al., 1999; Pirker et al., 2000; Moragues et al., 2000, 2002, 2003; Sinkkonen et al., 2000). Thus, evidence is tending toward all of them forming receptors in nature. In few cases are the possible heteropentameric subunit combinations known for these uncommon subunits (e.g., from immunoprecipitation studies using antibodies directed against γ1 or γ3 subunits) (Mossier et al., 1994; Quirk et al., 1994; Tögel et al., 1994) or θ subunits (Bonnert et al., 1999) and thus the total number of subtypes may be large or small. Each subunit γ1, γ3, ε, θ, and π makes an unknown number of combinations with α and β subunits, but probably will be slightly more than the minimum. In fact, the mere existence of these subunits has not been extended to show that the cells where they are expressed have any unique pharmacological or physiological properties produced from these subunits, although few serious searches have been attempted. Benzodiazepine insensitivity in the rat nucleus tractus solitarii correlates with ε expression (Kasparov et al., 2001), and prolongation of inhibitory postsynaptic currents recorded from cultured hypothalamic neurons has been shown to be negatively correlated with the detection of the ε subunit by RT-PCR (Sergeeva et al., 2005). In vitro recombinant studies suggest it should be possible to identify these rare subtypes if they exist. Because this has not been done yet, we decided to put these subtypes, combined with α and β, in the third category, C, tentative. Unusual subunit combinations of these minor subunits remain possible. The ε subunit has been reported to partner with other combinations in recombinant expression (Davies et al., 1997, 2001; Moragues et al., 2000; Wagner et al., 2005; Jones and Henderson, 2007) but whether this occurs in nature has not been sufficiently investigated (Bollan et al., 2008). As mentioned above, "pathological" (non-native) subunit compositions can be forced to occur in recombinant expression systems. The partnership association of the θ subunit has not been extensively studied (Bonnert et al., 1999; Sinkkonen et al., 2000) and, π, as a peripheral tissue constituent, has not been well characterized, although it forms channels in vitro with other GABA$_A$-R subunits (Hedblom and Kirk-

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TABLE 3
$GABA_A$ receptor native oligomer list

Category A: Identified
$\alpha1\beta2\gamma2$
$\alpha2\beta\gamma2$
$\alpha3\beta\gamma2$
$\alpha4\beta\gamma2$
$\alpha4\beta2\delta$
$\alpha4\beta3\delta$
$\alpha5\beta\gamma2$
$\alpha6\beta\gamma2$
$\alpha6\beta2\delta$
$\alpha6\beta3\delta$
ρ
Category B: Existence with high probability
$\alpha1\beta3\gamma2$
$\alpha1\beta\delta$
$\alpha5\beta3\gamma2$
$\alpha\beta1\gamma/\alpha\beta1\delta$
$\alpha\beta$
$\alpha1\alpha6\beta\gamma/\alpha1\alpha6\beta\delta$
Category C: Tentative
$\rho1$
$\rho2$
$\rho3$
$\alpha\beta\gamma1$
$\alpha\beta\gamma3$
$\alpha\beta\theta$
$\alpha\beta\epsilon$
$\alpha\beta\pi$
$\alpha x\alpha y\beta\gamma2$

ness, 1997; Neelands and Macdonald, 1999; Korpi and Sinkkonen, 2006). Some of these subunits can be shown to express more than one type of channel in recombinant expression work. In these cases, it is particularly important to evaluate recombinant studies with caution unless the nature of the subunit composition of the expressed receptors is unambiguously determined.

b. α/β Pentamers. Evidence has accumulated for the existence of receptors composed of α and β subunits, only. First, Bencsits et al. (1999) demonstrated that a large proportion of the $\alpha4$ receptors (approximately 50%) are not associated with γ or δ subunits and are possibly composed of α and β subunits only. This is in contrast with $\alpha1$ receptors. Second, in several knockout mice it was demonstrated that receptors composed of only α and β subunits do exist, for example, in $\gamma2$ knockout mice (Günther et al., 1995), δ knockout mice (Tretter et al., 2001), or $\alpha1$ knockout mice (Ogris et al., 2006). This suggests that such receptors might develop under other pathological conditions also. It is possible that such receptors arise during epilepsy, hormone treatment, ethanol intake, or during development. Localization studies show that in several brain regions α and β occur in the absence of γ and δ subunits. Either these subunits have other functions or combine with other subunits or they form receptors of α and β subunits only. Mortensen and Smart (2006) demonstrated by electrophysiological studies that there are extrasynaptic $\alpha\beta$ receptors on rat hippocampal pyramidal neurons. We could not find evidence for any specific α or any specific β subunit in these $\alpha\beta$ combinations or for the existence of more than one such subtype, so have placed one $\alpha\beta$ subtype in category B (Table 3).

c. Multiple α or β (other?) isoforms per pentamer. There is ample evidence from several different groups (Duggan et al., 1991; Lüddens et al., 1991; Khan et al., 1996; Sieghart and Sperk, 2002; Benke et al., 2004; reviewed in Whiting et al., 2000) for the coimmunoprecipitation of different α or β subunits, whereas different γ subunits in most studies were observed not to coimmunoprecipitate to a significant extent (for discussion, see Sieghart and Sperk, 2002). These findings suggest there is no unspecific aggregation of different receptor subtypes, and a variety of controls were performed to demonstrate specific coprecipitation (Jechlinger et al., 1998, Pöltl et al., 2003). Nusser et al. (1998) demonstrated separate locations but also colocalization of $\alpha1$ and $\alpha6$ in the cerebellum, suggesting the possibility that the two α subunits occur together in the same heteropentameric receptor. Benke et al. (2004) demonstrated the presence and abundance of $GABA_A$-Rs containing two different types of α subunits using point-mutated α subunits and reported on the abundance of these receptors in an in vivo system: The $\alpha1$ predominantly forms $\alpha1$-$\alpha1$, whereas other subunits predominantly form hetero-α. Finally, Minier and Sigel (2004) described the differential properties of receptors containing $\alpha1$ and $\alpha6$ subunits in a defined arrangement in a recombinant system. Each receptor configuration has its own pharmacological signature. We decided that the $\alpha1\alpha6\beta\gamma/\alpha1\alpha6\beta\delta$ qualified as a subtype for the list in category B. Furthermore, we placed an additional species of unknown subunit composition $\alpha x\alpha y\beta\gamma2$ in category C.

In addition, mixtures of β subunits probably do exist (Li and De Blas, 1997; Jechlinger et al., 1998), and two articles (Khan et al., 1994; Quirk et al., 1994) suggested that two kinds of γ subunit could be included. However, other researchers could not confirm the latter finding (Jechlinger et al., 1998; Pöltl et al., 2003).

d. Receptors containing 1, 2, 4, or 5 different subunits. The evidence for other combinations of subunits with other than three kinds of subunit (homo-oligomeric $\beta3$ subunit channels and hetero-oligomeric channels containing 2, 4, or 5 different subunits), except ρ and $\alpha\beta$ as discussed, is limited to recombinant expression work and to some reports indicating the existence of two different α or two different β subunits in the same native $GABA_A$-R. None currently qualify for inclusion within the list (see discussion in Sieghart and Sperk, 2002).

2. Localization, Trafficking, Post-Translational Modifications, and Associated Proteins. Although these factors could alter receptor pharmacology (Kittler and Moss, 2003), little is known about such factors, no in vivo function has been identified unequivocally, and thus, none are accepted at this time.

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D. Tentative List of Naturally Occurring Receptor Subtypes

Three categories of entries are listed: identified, existence with high probability, and tentative.

IV. Concluding Remarks

This classification starts with the 19 human GABA$_A$-R genes (Table 1) and some evidence for the likely subunit combination and stoichiometry of the abundant $(\alpha1)_2(\beta2)_2\gamma2$ heteropentameric subtypes. Because of the emphasis of IUPHAR on the human clinic, the present discussion is limited to the human, rat, and mouse, acknowledging that GABA$_A$-Rs exist in all organisms with a nervous system, including invertebrates. We further made the decision to concentrate on the usual location of GABA$_A$-Rs, the central nervous system, realizing that there is expression of functional receptors in peripheral tissues.

In attempting to define native subtypes, we propose (Table 2) five criteria (two for recombinant studies and three for native studies) for inclusion of a given subunit combination on the list. We note that even for the 19 subunit genes, the evidence for some subunits participating in native subtypes varies considerably. Whereas there is some evidence that each of the 19 exists in at least one subtype, some of them meet few of the five criteria. We decided to use three different categories in making the list, consistent with the work in progress nature of such a list (Table 3). This is likewise the situation for the other members of the LGIC receptor superfamilies. The categories in the list are identified, existence with high probability, and tentative. We added the second category, existence with high probability, for those candidates that meet some but not a sufficient number of the criteria summarized here, allowing the reader to decide whether the evidence is convincing. Because the existence of native receptors containing the minor subunits did not meet even the criteria for this category but are likely to exist in at least one subtype each, we added the third category, tentative. One can see that each entry in the tentative category might actually exist as multiple discrete subtypes, and thus the number on the list will expand in the future, possibly by a substantial number, as suggested by Barnard et al. (1998). Our final list (Table 3) includes 11 subtypes in category A. We have 6 entries in category B. We placed 9 in category C, and if they all stay, that makes 26 in 2008, a number that is certain to increase over time.

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