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$GABA_A$ Receptors: Properties and Trafficking

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ABSTRACT Fast synaptic inhibition in the brain and spinal cord is mediated largely by ionotropic γ-aminobutyric acid (GABA) receptors. $GABA_A$ receptors play a key role in controlling neuronal activity; thus modulating their function will have important consequences for neuronal excitation. $GABA_A$ receptors are important therapeutic targets for a range of sedative, anxiolytic, and hypnotic agents and are involved in a number of CNS diseases, including sleep disturbances, anxiety, premenstrual syndrome, alcoholism, muscle spasms, Alzheimer's disease, chronic pain, schizophrenia, bipolar affective disorders, and epilepsy. This review focuses on the functional and pharmacological properties of $GABA_A$ receptors and trafficking as an essential mechanism underlying the dynamic regulation of synaptic strength.

KEYWORDS gamma-aminobutyric acid, benzodiazepines, ligand-gated ion channels, clathrin, gephyrin, GABARAP, Plic-1, endocytosis

INTRODUCTION

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS). The inhibitory effect of GABA is mediated either by $GABA_A$ receptors, which are ionotropic GABA-gated chloride channel receptors, or by the metabotropic $GABA_B$ receptors. This review focuses on $GABA_A$ receptors. $GABA_A$ receptors are responsible for fast inhibitory neurotransmission in the adult CNS. These receptors are members of the nicotinicoid superfamily of ligand-gated ion channels, a family that includes nicotinic acetylcholine receptors, glycine receptors, and the $5HT_3$ serotonin receptors. Members of the superfamily share significant sequence similarity and are believed to have a similar structure with a large N-terminal extracellular region, including a highly conserved extracellular Cys-Cys loop. Direct modulators of $GABA_A$ receptors include benzodiazepines, barbiturates, neurosteroids and anesthetics. The assembly of $GABA_A$ receptors in neuronal membranes is regulated by different mechanisms, including phosphorylation (Brandon and Moss, 2000; Moss and Smart, 2001) and ubiquitination (DiAntonio and Hicke, 2004; Luscher and Keller, 2004), both of which have been reviewed previously.

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STRUCTURE AND FUNCTION OF THE $GABA_A$ RECEPTORS

Structure of $GABA_A$ Receptors

$GABA_A$ receptors are members of the superfamily of ligand-gated ion channels that gate Cl^- and–to a lesser extent–HCO_3^-. They are usually found on postsynaptic sites of neurons. The first $GABA_A$ receptor was isolated and sequenced in 1987 (Schofield *et al.*, 1987). $GABA_A$ receptors are heteromeric pentamers composed of five subunits that can belong to different subfamilies. To date, 19 different subunits have been isolated: α1-6, β1-3, γ1-3, δ, ε, π, ρ1-3, and θ. Additional structural complexity exists due to alternative splicing of subunits, notably the γ2 subunits that exist in a short (γ2S) and a long subunit (γ2L) and distinguished by additional eight amino acids (Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys) in the intracellular loop region of the γ2 long form. Splice variants have also been detected for other subunits. Each subunit consists of a short extracellular C-terminus, a large extracellular N-terminus, four α-helical transmembrane domains (TM1-TM4) and a large variable-sized cytoplasmic loop between TM3 and TM4 (Figure 1). The intracellular loop contributes most of the cytoplasmic domain of the $GABA_A$ receptor and includes multiple protein-protein interaction sites for putative trafficking and both postsynaptic scaffold proteins and phosphorylation sites for diverse serine, threonine and tyrosine kinases. The amphiphilic TM2 domain provides the lining of the ion pore (~6 Å) within the pentameric structure. The molecular weight of the $GABA_A$ receptor complex is estimated to be approximately ~300 kDa (Table 1). The expression of $GABA_A$ receptor subtypes is spatially, regionally and developmentally regulated, with individual subunits having distinct but overlapping expression patterns (Fritschy and Mohler, 1995; Laurie *et al.*, 1992). In addition to differential subunit expression throughout brain regions, the $GABA_A$ receptor subunit composition varies between cell types and undergoes differential subcellular targeting (Table 1); for example, $GABA_A$ receptors containing α5 subunits are localized mainly in the soma, dendrites, and axons of hippocampal neurons, while α2 subunits are found to be concentrated

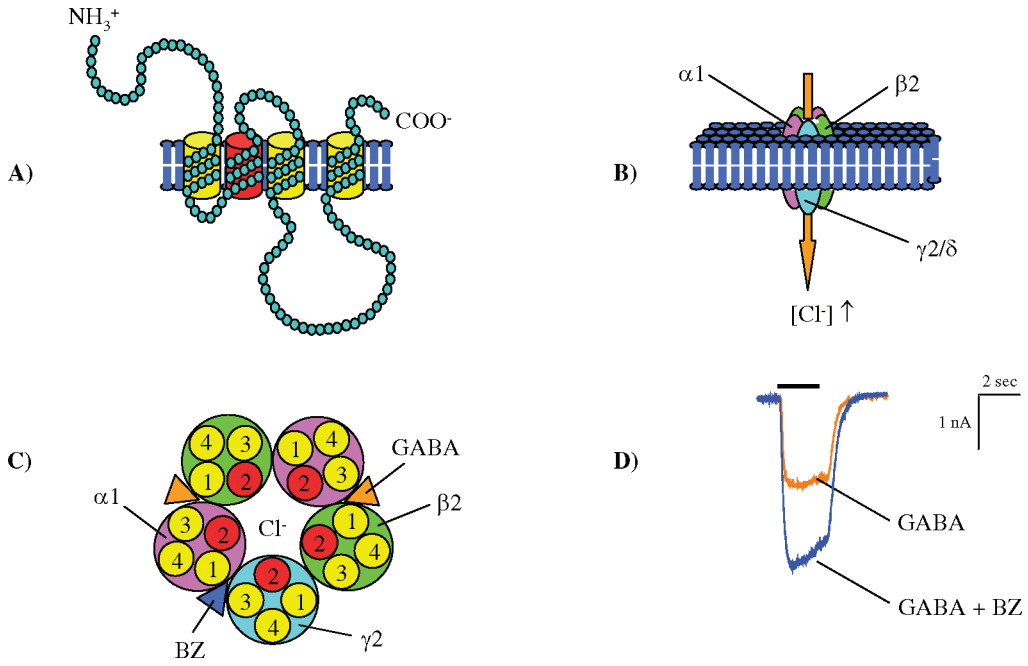

Figure 1 Structure and function of the $GABA_A$ receptor. (*A*) $GABA_A$ receptor subunit composition. Receptor subunits consist of four hydrophobic transmembrane domains (TM1-4), where TM2 is believed to line the pore of the channel. The large extracellular N-terminus is the site for ligand binding as well as the site of action of various drugs. Each receptor subunit also contains a large intracellular domain between TM3 and TM4, which is the site for various protein-protein interactions as well as the site for post-translational modifications that modulate receptor activity. (*B*) Five subunits from seven subunit subfamilies assemble to form a heteropentameric chloride permeable channel. Despite the extensive heterogeneity of $GABA_A$ receptors most synaptic receptors are thought to consist of 2α, 2β and 1γ/1δ subunit. γ2-subunit containing $GABA_A$ receptors display a tendency to localize at synaptic sites, while δ-subunit containing $GABA_A$ receptors are found predominantly at extrasynaptic sites. (*C*) Stoichiometry and subunit arrangement of the $GABA_A$ receptor. (*D*) Benzodiazepine effect on the $GABA_A$ receptor. Whole cell GABA-gated current response to 5 μM GABA application alone and coapplication with benzodiazepine (BZ: 100 nM flurazepam) at a holding potential of –50 mV.

TABLE 1 **Overview of the major $GABA_A$ receptor subunits**

$GABA_A$ receptor subunit	Mw (kDa)	Preferential regional distribution (cellular localization)	Specific pharmacological properties
$\alpha 1$	51	Cerebral and cerebellar cortex, thalamus, pallidum (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Sedative, amnesic and anticonvulsant action of BZ (Rudolph *et al.*, 1999)
$\alpha 2$	52	Hippocampus, amygdaloidal nucleus, striatum (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Anxiolytic and myorelaxant action (at higher initial doses) of BZ (Crestani *et al.*, 2001; Low *et al.*, 2000)
$\alpha 3$	53	Brainstem (noradrenergic and serotonergic neurons), basal forebrain (cholinergic neurons), thalamus (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Anxiolytic and myorelaxant (at high doses) action of BZ (Crestani *et al.*, 2001)
$\alpha 4$	60–66	Thalamus, striatum, dentate gyrus (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Ethanol sensitivity (Wallner *et al.*, 2003)
$\alpha 5$	53–56	Hippocampus (CA1 region) (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden, *et al.*, 1992)	Amnesic and myorelaxant action of BZ, memory enhancement (Rudolph and Mohler, 2006)
$\alpha 6$	53–56	Cerebellum (granular cell layer) (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Ethanol sensitivity (Wallner *et al.*, 2003)
$\beta 1$	54–56	Hippocampus (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Salicylidene salicylhydrazide as selective inhibitor (Thompson *et al.*, 2004)
$\beta 2$	55–57	Thalamus (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Anesthetic action of etomidate (Rudolph and Antkowiak, 2004)
$\beta 3$	54–56	Striatum, hippocampus, cerebellum (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	Anesthetic action of propofol and etomidate (Quinlan *et al.*, 1998; Rudolph and Antkowiak, 2004)
$\gamma 1$	45–51	Pallidum, central and medial amygdaloid nuclei, substantia nigra (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	No specific properties
$\gamma 2$	45–51	Ubiquitous in the brain (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	No specific properties
$\gamma 3$	43–46	Cerebral cortex (Pirker *et al.*, 2000; Sieghart and Sperk, 2002; Wisden *et al.*, 1992)	No specific properties
δ	45–53	Cerebellum (granular cell layer), thalamus, striatum, dentate gyrus (Pirker, *et al.*, 2000; Sieghart and Sperk, 2002; Wisden, *et al.*, 1992)	Ethanol and neurosteroid sensitivity (Belelli and Lambert, 2005; Wallner *et al.*, 2003)

Mw = molecular weight; AIS = axon initial segment, BZ = benzodiazepine effects.

in the axon initial segment (Fritschy *et al.*, 1998; Nusser *et al.*, 1996). The $\alpha 4$, $\alpha 5$, $\alpha 6$, and δ-containing subunits are located extrasynaptically and are responsible for the tonic (persistent: long-term GABA exposure at low concentrations) inhibitory current (Caraiscos *et al.*, 2004), while all of the other $GABA_A$ receptor subunits–especially the $\gamma 2$-subunit containing $GABA_A$ receptors–are preferentially expressed on the synaptic site and are involved in phasic (transient: short-term GABA exposure at high concentrations) inhibition (Farrant and Nusser, 2005). As a result of their distinct subunit composition, extrasynaptic $GABA_A$ receptors have different pharmacological and kinetic properties from those of synaptic $GABA_A$ receptors (Korpi and Sinkkonen, 2006). Furthermore, extrasynaptic receptors are proposed to be an important target for certain

general anesthetics, neurosteroids, and alcohol (Wallner *et al.*, 2003; Wei *et al.*, 2004).

The most important findings on the $GABA_A$ receptor structure have been made from recombinant $GABA_A$ receptor expression studies. Coexpression of the α and the β subunits in heterologous cells is sufficient for assembly of a functional $GABA_A$ receptor, but such a receptor has a low single-channel conductance (12 pS, normally 25 to 30 pS) and has no benzodiazepine sensitivity (Lorez *et al.*, 2000). $\beta 1$ and $\beta 3$ subunits can also spontaneously form open Cl^- channels that not only can be modulated by GABA but are also inhibited by picrotoxin and actived by pentobarbital (Connolly *et al.*, 1996; Krishek *et al.*, 1996). However, expression of the $\gamma 2S$ subunit alone does not result in the formation of functional channels, consistent with the suggestion that it can access the cell surface as a monomer (Connolly *et al.*, 1999). Subunits expressed alone (α, β, or γ subunits) do not form GABA-gated Cl^- ion channels because they are retained within the endoplasmic reticulum, from where they are rapidly degraded (Connolly *et al.*, 1996; Pritchett *et al.*, 1989; Shivers *et al.*, 1989). The majority of $GABA_A$ receptor subtypes in the brain contain diverse α and β subunit variants in combination with the γ or δ subunit. $GABA_A$ receptors that contain the γ subunit are usually located on synaptic sites, whereas receptors assembled by the δ subunit are typically located extrasynaptically (Table 1). The δ subunit is thought to be assembled only in $GABA_A$ receptors that contain $\alpha 6$ in the cerebellum and $\alpha 4$ subunits in the forebrain, respectively (Laurie *et al.*, 1992; Pirker *et al.*, 2000; Wisden *et al.*, 1992). The subunit stoichiometry of native $GABA_A$ receptors has not been cleary determined, but the established consensus, based on recombinant receptor studies, favors the existence of pentamers composed of 2α, 2β and $1\gamma 2$ subunits (Chang *et al.*, 1996; Tretter *et al.*, 1997). The most abundant receptor combinations have been demonstrated using immunolabelling and autoradiography: $\alpha 1\beta 2\gamma 2$ (~60%), $\alpha 2\beta 3\gamma 2$ (~15%) and $\alpha 3\beta 3\gamma 2$ (~10%). The subunit combinations $\alpha 4\beta 2\gamma / \alpha 4\beta n\delta$, $\alpha 5\beta 1/3\gamma 2$, $\alpha 6\beta 2/3\gamma 2$ and $\alpha 6\beta n\delta$ each represent less than 5% of all $GABA_A$ receptors in the brain (McKernan and Whiting, 1996; Rudolph and Antkowiak, 2004). It should be noted that the relative and absolute amounts of receptor subtypes are not known exactly. The δ, ε, and π subunits of $GABA_A$ receptors are believed to be substitute partners for the $\gamma 2$ subunit (Davies *et al.*, 1997; Shivers *et al.*, 1989), whereas the θ subunit might be able to replace the β subunit (Bonnert *et al.*, 1999). The distinct class of $\rho 1$–3 subunits, which are found mainly in neurons of the retina and expressed as hetero- or homo-oligomers, have been classified as a specialized set of $GABA_A$ receptors and known as $GABA_{A0r}$ receptors. In contrast to $GABA_A$ receptors, which are sensitive to bicuculline, and $GABA_B$ receptors, which are sensitive to baclofen, $GABA_{A0r}$ receptors are insensitive to either drugs (Table 2).

Function of $GABA_A$ Receptors

Different $GABA_A$ receptor subunits are arranged to form a Cl^- selective ligand-gated ion channel with distinct biophysical and pharmacological properties (Brickley *et al.*, 1999; Fisher and Macdonald, 1997). When GABA binds extracellulary between the α and the β subunit (approximately at the site of benzodiazepine receptor), it acts as an agonist, inducing conformational changes of the $GABA_A$ receptor complex, which increases the permeability of the central ion pore to Cl^- ions. Once GABA is removed by glia or by presynaptic terminals, the channel comes to a closed state and can–after desensitization–thus be re-opened. The influx of Cl^- and HCO_3^- ions hyperpolarizes (or depolarizes) the cell, decreasing the likelihood of the neuron firing an action potential and producing a general inhibitory effect on neuronal activity. This action may explain the sedative and anticonvulsant effects of $GABA_A$ receptors, such as those caused by benzodiazepines, anesthetics, neurosteroids, or alcohol. While intravenous anesthetics,

TABLE 2 **Pharmacological characterization of different GABA receptors**

GABA receptors	Receptor type	Agonist	Antagonist
$GABA_A$ receptor	Ionotropic	Muscimol, isoguvacine	Bicuculline, gabazine
$GABA_B$ receptor	Metabotropic	(R)-(−)-baclofen, CGP35024	CGP35348
$GABA_{A0r}$ receptor	Ionotropic	CACA	TPMPA

CGP35024 = 3-aminopropyl-(P-methyl)-phosphinic acid, CGP35348 = p-(3-aminopropyl)-P-diethoxymethylphosphinic acid, CACA = cis-4-aminocrotonic acid, TPMPA = (1,2,5,6-tetrahydropyridin-4-yl)-methylphosphinic acid.

such as barbiturates, and volatile anesthetics, interact with multiple targets (multiple receptor theory), benzodiazepines act primarily as positive allosteric modulators of the $GABA_A$ receptor. To maintain the "physiological" hyperpolarizing GABA effect it is necessary to retain a stable Cl^- gradient by Cl^- pumps of various ion transporters. One example is the CNS specific electroneutral K^+-Cl^--cotransporter type 2, named KCC2, whose expression is, like that of $GABA_A$ receptors, spatially, regionally, and developmentally regulated and whose properties allow the regulation of $[Cl^-]_i$ and $[K^+]_o$ in neurons to maintain electrochemical gradients for hyperpolarizing GABAergic inhibition. The direction of the Cl^- flux, inward or outward, through the $GABA_A$ receptor Cl^--conducting pore depends on the Cl^- gradient across the plasma membrane. In the axon initial segment (AIS) and in the early stage in neuronal development, KCC2 is not expressed and the internal Cl^- concentration is high (Lu *et al.*, 1999). In this situation, the switch in the Cl^- equilibrium and the shift of the transmembrane potential can lead to a Cl^- efflux, which results in a depolarizing response by activating voltage-gated Ca^{2+} channels (Chavas *et al.*, 2004; Vardi *et al.*, 2000). This process is further complicated because of the additional HCO_3^- permeability of the $GABA_A$ "mixed" anion channel and the presence of additional ion transporters such as the HCO_3^-/Cl-exchanger or the Na^+-K^+-$2Cl^-$ cotransporter (Jarolimek *et al.*, 1999; Kaila, 1994). Thus GABA operates mainly as an excitatory transmitter on immature neurons and as an inhibitory transmitter during maturation. The physiological relevance of these different $GABA_A$ receptor responses, especially in mature neurons, is difficult to address and remain still unclear.

Pharmacology of $GABA_A$ Receptors

Benzodiazepines are characterized by their anxiolytic, anticonvulsant, sedative, muscle relaxative and amnesic effects. They enhance the GABA-induced Cl^- current via allosteric modulation, increasing the affinity of GABA for Cl^- channel opening and therefore the "open frequency probability" of these channels, while barbiturates prolong the "lifetime of the open states." The action of benzodiazepines thus differs markedly from that of barbiturates, and has clinical importance in drug intoxication. Barbiturates have a propensity to be fatal in overdose, probably because of their direct action on $GABA_A$ receptors. Benzodiazepines are safer, perhaps because vital brain circuits cannot be inhibited over and above the level that would be achieved by natural GABAergic effects. While benzodiazepine binding requires an additional $\gamma 2$ subunit for functional modulation of $GABA_A$ Cl^- channels (Pritchett *et al.*, 1989; Sigel *et al.*, 1990) barbiturates and neurosteroids seem to have little subtype specificity.

The binding pocket for benzodiazepines is thought to be located at the interface between the α and the γ subunit in the extracellular N-terminal portion of the receptor subunits. These binding sites are heterogeneous and, since there are 6 different α and 3 different γ subunits, up to 18 different central benzodiazepine binding sites exist. The effect of benzodiazepine is highly dependent on $GABA_A$ receptor assembly. Those $GABA_A$ receptors containing an α1-3 or α5 subunit in combination with any of the β and $\gamma 2$ subunits have been pharmacologically classified as "diazepam-sensitive," while those $GABA_A$ receptors containing an α4 or α6 subunit–receptors that do not recognize the classical benzodiazepine agonists such as diazepam or flunitrazepam–are referred as "diazepam-insensitive" (Benson *et al.*, 1998; Luddens *et al.*, 1990; Malminiemi and Korpi, 1989). The diazepam-sensitive receptors can be further subdivided by their sensitivity to benzodiazepines such as quazepam, 2-oxoquazepam, β-carboline derivates, imidazopyridines, or triazolopyridazine with the higher-affinity receptors (type-I or BZ-I receptors) containing α1 subunits and the lower-affinity receptors (BZ-II receptors) containing α2, α3 or α5 subunits (Sieghart, 1989). Some benzodiazepines interact preferentially with $GABA_A$ receptors that contain α1 or α5 subunits (Sieghart, 1995); for example, ligands with preferential affinity for the α1-subunit containing $GABA_A$ receptors, such as zolpidem or zaleplon, are used clinically as sedative-hypnotics (Mohammadi *et al.*, 2006). A large number of amino acid residues have been identified both on α and γ subunits as main members of the benzodiazepine binding pocket. The Histidine 101 (His101) on the α1 subunit in the extracellular domain has been identified as the major target of [^{3}H]-flunitrazepam (Duncalfe *et al.*, 1996). In contrast, the diazepam-insensitive $GABA_A$ receptor subtypes (α4 or α6) have an arginine in the corresponding position. Rudolph and colleagues demonstrated that point-mutated α1(His101Arg) mice fail to show the sedative, amnesic and partly the anticonvulsant effect of diazepam (Rudolph *et al.*, 1999), whereas the anxiolytic,

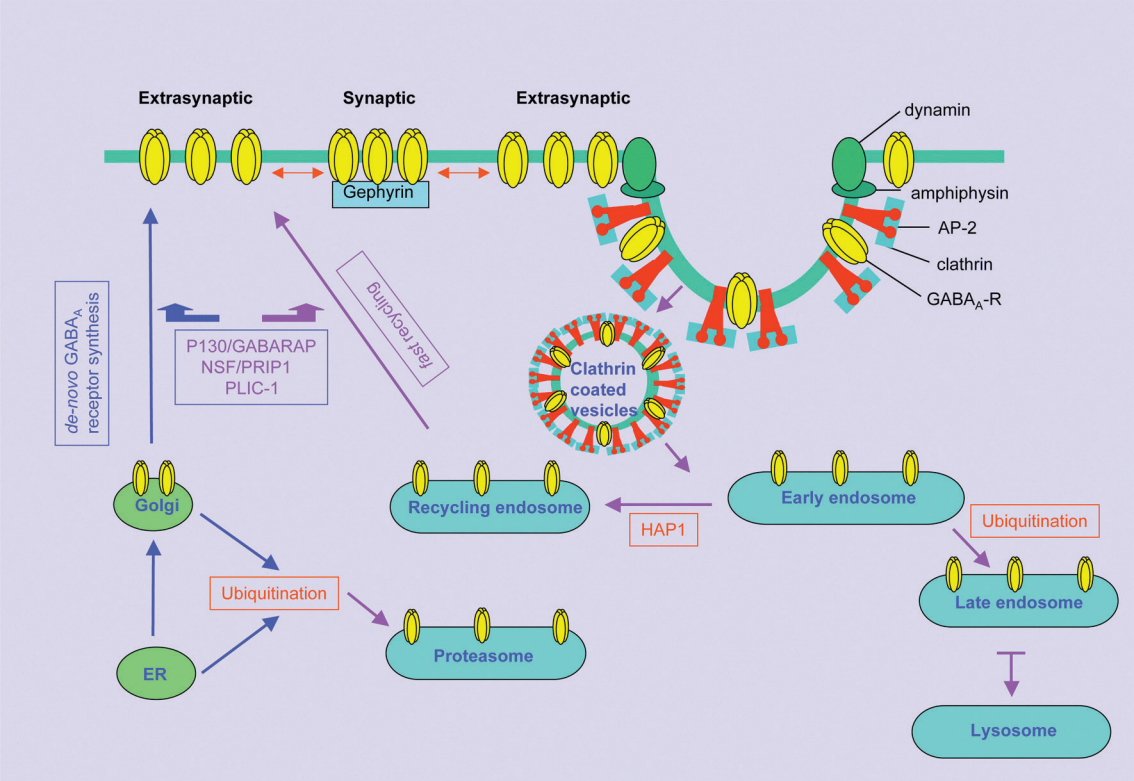

Figure 2 **Trafficking of GABA$_A$ receptors. Exocytosis of GABA$_A$ receptors: GABA$_A$ receptors are assembled in the endoplasmic reticulum and exit into the Golgi complex, a process thought to be mediated by the guanidine exchange factor BIG2. The microtubule binding protein GABARAP associates with the γ2 subunit of GABA$_A$ receptors at the Golgi and aids in trafficking to the plasma membrane. In addition, the ubiquitin-like protein Plic-1 is localized in intracellular compartments where it binds α and β subunits. Binding of Plic-1 to the GABA$_A$ receptor is thought to regulate membrane targeting by preventing degradation in the proteasome. GABA$_A$ receptors are thought to be inserted into the plasma membrane at extrasynaptic sites where they are allowed to diffuse into synaptic sites and are clustered there with the scaffolding protein gephyrin. Endocytosis of GABA$_A$ receptors: GABA$_A$ receptors have been shown to undergo constitutive clathrin-mediated endocytosis. The intracellular loops of β and γ subunits are known to interact with the clathrin adaptor protein AP2. Upon internalization GABA$_A$ receptors are transported to sorting endosomes where they can be sorted either for recycling back to the plasma membrane or for degradation (lysosomal/proteasomal). An interaction between β subunits and the endosomal protein HAP1 is thought to facilitate the recycling of receptors back to the plasma membrane. The blue arrows represent trafficking events in the secretory pathway while the mauve arrows indicate events in the endocytic events.**

myorelaxant, and ethanol-potentiating effects were fully retained, indicating that these effects are mediated by the non-mutated GABA$_A$ receptors containing α2–3 or α5 subunits (Table 1). Confirming this study, the anxiolytic action of diazepam was absent in mice with α2(H101R) point mutation but present in mice with the α3(H126R) point mutation, indicating that the α2 subunit is a highly specific target for the development of selective anxiolytic drugs (Low *et al.*, 2000).

TRAFFICKING OF GABA$_A$ RECEPTORS

The number of GABA$_A$ receptors expressed on the cell surface is central to the control of neuronal inhibition. GABA$_A$ receptors are not static entities in neuronal plasma membranes but undergo rapid movement into (exocytosis) and out of (endocytosis) these structures. Modifications of GABA$_A$ receptor cell surface number underlie changes in inhibitory postsynaptic current amplitude (Kittler *et al.*, 2004). To maintain a stable cell-surface receptor number, continual membrane insertion of *de novo* synthesized or recycled GABA$_A$ is required (Figure 2).

Exocytosis of GABA$_A$ Receptors

Individual GABA$_A$ receptors, when synthesized *de novo*, co-oligomerize in the endoplasmatic reticulum membrane, in association with the chaperone proteins BiP (heavy chain binding protein), calnexin or BIG-2, and assemble into receptor complexes (Charych *et al.*, 2004; Connolly *et al.*, 1996; Wisden and Moss, 1997). *BIG-2* (brefeldin A-inhibited GDP/GTP exchange factor 2), a Sec7 domain-containing guanine

nucleotide exchange factor known to be involved in vesicular and protein trafficking through its interaction with the β subunit of $GABA_A$ receptor, promotes the translocation of assembled $GABA_A$ receptors from the endoplasmatic reticulum via the Golgi apparatus to the plasma membrane. Unassembled $GABA_A$ receptor subunits are degraded by the lysosomal pathway. The cell surface receptors may aggregate to form either synaptic or extrasynaptic clusters.

Endocytosis, Clustering, and Trafficking of $GABA_A$ Receptors

Endocytosis is defined as the internalization of plasma membrane proteins, receptors, ion channels, and/or extracellular molecules into cells in membrane-bound vesicles. Dynamin-dependent endocytosis is important in the regulation of cell surface levels of a number of integral membrane proteins (Schmid, 1997). For $GABA_A$ receptors, clathrin-mediated endocytosis is the major mechanism of receptor internalization (Kittler *et al.*, 2000). In addition to clathrin-dependent endocytosis of these receptors, a clathrin-independent pathway has also been demonstrated in HEK293 cells (Cinar and Barnes, 2001). Although the detailed mechanism of the clathrin-independent endocytosis of $GABA_A$ receptors is still unclear, processes such as ubiquitin-dependent or caveolin/lipid-raft-dependent pathways are likely involved (Bedford *et al.*, 2001; DiAntonio and Hicke, 2004; Le Roy and Wrana, 2005; Nichols, 2002).

Based on structural studies, clathrin-dynamin mediated endocytosis has been subdivided into different stages: (a) clathrin-coating, (b) vesicle invagination, (c) fission (with dynamin and amphiphysin), (d) movement of vesicles into the cell interior, (e) vesicle uncoating, (f) fusion with early or sorting endosomes, and (g) recycling to the plasma membrane or (h) lysosomal degradation. When an adaptor protein (AP) such as AP2 binds to the cytoplasmatic domains of the receptor, it recruits clathrin and promotes the assembly of the clathrin triskelion into a clathrin coat on the inner surface of the plasma membrane. The $\mu 2$ subunit of the AP2 in particular has the ability to bind a number of endocytosis auxiliary proteins such as AP180, epsin, amphiphysin and auxilin. The β-adaptin subunit of the adaptor protein complex AP2, clathrin, the GTPase dynamin, and its binding partner amphiphysin are key elements of the endocytotic machinery (Marsh and McMahon, 1999; Takei *et al.*, 1999). Endocytosed receptor cargo first appears in early or "sorting" endosomes, which are the relay stations along this pathway. From there the cargo can be sorted for degradation in late endosomes by lysosomal proteases, ubiquitinated by proteasomes or recycled back to the plasma membrane (Figure 2). There are two major pathways for $GABA_A$ receptor recycling: (a) a fast pathway from recycling endosomes, or (b) a slow pathway from late endosomes via the Golgi apparatus (Connolly *et al.*, 1999; Kittler *et al.*, 2004).

Under basal conditions, $GABA_A$ receptors undergo clathrin-dependent endocytosis (Herring *et al.*, 2003; Kittler *et al.*, 2005, 2000). Blocking this clathrin-dependent endocytosis with P4 peptide disrupts the association between amphiphysin and dynamin, causing large sustained increases in the amplitude of miniature inhibitory postsynaptic currents (mIPSCs). This suggests that $GABA_A$ receptors constantly (re)-cycle between cell surface and endosomal compartments (Kittler *et al.*, 2000). The intracellular loops of β and γ subunits of the $GABA_A$ receptor are the major interacting partners of the adaptor complex AP2, which is critical for the recruitment of integral membrane proteins into clathrin-coated pits. Two major classes of endosomal sorting signals have been identified: (a) tyrosine (Y)-based, and (b) dileucine (LL)-based signals, which interact with the AP2 complex (Marsh and McMahon, 1999). The AP2 adaptin "dileucine-motif" within the $\beta 2$ subunit (LL 343/344) seems to be important for the internalization of $GABA_A$ receptors (Herring *et al.*, 2003). Evidence for the crucial role of the $\gamma 2$ subunit in regulating $GABA_A$ receptor synpatic targeting comes from studies in which the deletion of the $\gamma 2$ subunit produces a 70% decrease in $GABA_A$ number at inhibitory synapses and leads to a loss in synaptic staining of the tubulin-bridging molecule gephyrin (Essrich *et al.*, 1998; Kneussel *et al.*, 1999). Several proteins have been identified that bind directly to $GABA_A$ receptors to regulate their trafficking (Table 3). More recently the yeast two-hybrid system has been used to identify such $GABA_A$ receptor-associated proteins as GABARAP and Plic-1.

GABARAP, a $GABA_A$-receptor-associated and microtubule-associated protein, is a member of a protein family that includes MAP-LC3 (microtubule-associated protein light chain 3), GATE-16 (Golgi-associated ATPase enhancer), and Apg8/Aut7, a yeast ortholog of the mammalian factors GABARAP and

TABLE 3 **$GABA_A$ receptor associated proteins and their function in trafficking**

$GABA_A$ receptor trafficking proteins	Mw (kDa)	Function in trafficking and/or cell surface stability
AP2	110	Clathrin-dynamin mediated endocytosis of $GABA_A$ receptor (Kittler *et al.*, 2005, 2000)
BIG-2	200	$GABA_A$ receptor exocytosis: post-Golgi apparatus vesicular trafficking of assembled $GABA_A$ receptors (Charych *et al.*, 2004; Connolly *et al.*, 1996)
GABARAP	17	$GABA_A$ receptor clustering and targeting (Wang *et al.*, 1999)
gephyrin	93	$GABA_A$ receptor clustering and targeting (Essrich *et al.*, 1998)
GODZ	34	$GABA_A$ receptor exocytosis: posttranslational palmitoylation of γ2-subunit containing $GABA_A$ receptors (Keller *et al.*, 2004)
GRIF-1	115	Anterograde transport of $GABA_A$ receptor (Beck *et al.*, 2002)
GRIP-1	100	Anterograde transport of $GABA_A$ receptor (Kittler *et al.*, 2004)
HAP1	68	Endocytic sorting and vesicular trafficking: inhibits degradation of internalized $GABA_A$ receptor (Kittler *et al.*, 2004)
NSF	76	Vesicular trafficking (Kittler *et al.*, 2001)
Plic-1	67	Stabilization of cell surface $GABA_A$ receptors (Bedford *et al.*, 2001)
PRIP-1	130	Intracellular $GABA_A$ receptor trafficking (Kanematsu *et al.*, 2005)
ULK-1	120	Vesicle transport and axonal elongation (Okazaki *et al.*, 2000)
Unc-18	67	Vesicular trafficking: facilitator of vesicle docking (Weimer *et al.*, 2003)
Trak-1	106	Intracellular $GABA_A$ receptor trafficking (Gilbert *et al.*, 2006)

BIG-2 = brefeldin A-inhibited GDP/GTP exchange factor 2; GABARAP = $GABA_A$-receptor-associated protein; GODZ = Golgi-specific DHHC zinc finger protein; GRIF-1 = $GABA_A$ receptor interacting factor-1; GRIP-1 = glutamate receptor interacting protein 1; HAP1 = Huntingtin associated protein-1; NSF = N-ethylmaleimide sensitive factor; Plic-1 = protein linking IAP [integrin-associated protein] with cytoskeleton-1; PRIP-1 = Phospholipase-C related catalytically inactive protein type-1; Trak-1 = trafficking kinesin binding protein-1; ULK-1 = Unc [uncoordinated]-51-like [serine/thyreonine] kinase; Unc-18 = uncoordinated protein 18.

GATE-16 (Kabeya *et al.*, 2000; Sagiv *et al.*, 2000; Tanida *et al.*, 2003). GABARAP is enriched predominantly in intracellular membranes including the Golgi apparatus and postsynaptic cisternae. It is not found at significant levels within inhibitory synapses (Kittler *et al.*, 2001). Functionally, GABARAP acts as a linker protein between the microtubule protein tubulin and the intracellular loop of the γ2 subunit, which promotes the clustering of γ2-subunit containing $GABA_A$ receptors (Wang *et al.*, 1999). In addition, GABARAP has a basic N-terminus that can bind to tubulin and a ubiquitin-like C-terminal γ2 subunit-binding region. Additional binding partners of this multifunctional adapter molecule include gephyrin, GRIF-1, NSF, PRIP-1, and ULK1.

Gephyrin was originally found to anchor glycine receptors to the subsynaptic cytoskeleton (Prior *et al.*, 1992). Like GABARAP gephyrin is also a tubulin-binding protein involved in organizing postsynaptic $GABA_A$ receptors at inhibitory GABAergic synapses. This provides a structural link between the subsynaptic compartment and the cytoskeleton. Gephyrin is concentrated in the postsynaptic membrane at many inhibitory synapses. Studies of the mechanisms for clustering of major $GABA_A$ receptor subclasses at GABA-dependent synapses have demonstrated that both the γ2 subunit of $GABA_A$ receptors and gephyrin are required in receptor clustering, targeting and localization (Essrich *et al.*, 1998). Although previous studies suggested that a linker protein mediates the receptor-gephyrin interaction, recent pulldown experiments demonstrate a direct interaction with $GABA_A$ receptor subunits (Tretter VT and Moss SJ, unpublished observations). One study also reveals that synaptic $GABA_A$ receptors have lower levels of lateral mobility as compared to their extrasynaptic counterparts, and suggests a specific role for gephyrin in reducing the diffusion of $GABA_A$ receptors, facilitating their anchoring at inhibitory synapses (Jacob *et al.*, 2005).

GRIP-1 (glutamate receptor interacting protein 1), which was first found in the glutamatergic system, also interacts with the γ2 subunit of $GABA_A$ receptors. However, its role at inhibitory synapses remains unknown, although its ability to interact with GABARAP suggests that it may be involved in the synaptogenesis of inhibitory synapses or in the regulation of $GABA_A$ receptor function (Dong *et al.*, 1997; Kittler *et al.*, 2001; Kittler *et al.*, 2004; Li *et al.*, 2005).

N-ethylmaleimide-sensitive factor (NSF), an ATPase and chaperone for SNARE (soluble N-ethylmaleimide-

sensitive factor attachment protein receptor), is a necessary cofactor for vesicle-mediated Golgi transport. NSF plays an important role in intracellular membrane trafficking and fusion events, and mediates the intracellular transport of $GABA_A$ receptors (Kittler *et al.*, 2001). NSF interacts with residues of the β subunits of $GABA_A$ receptors (Goto *et al.*, 2005).

PRIP-1 (Phospholipase-C related catalytically inactive protein type-1) or previous called *p130* (protein 130) is an inositol 1,4,5-trisphosphate-binding protein with a molecular mass of 130 kDa and a domain organization similar to that of phospholipase C-δ1 but lacking PLC activity. PRIP-1 competitively inhibits the binding of the γ2 subunit of $GABA_A$ receptors to GABARAP, suggesting that this protein participates in $GABA_A$ receptor assembly and transport to the cell surface (Kanematsu *et al.*, 2002; Kanematsu *et al.*, 2005). Furthermore, this protein is involved in the phospho-regulation of $GABA_A$ receptors (Terunuma *et al.*, 2004).

ULK1 (Unc-51-like kinase), which interacts with GATE-16 and GABARAP, essential for intra-Golgi transport, seems to be important for vesicle transport and axonal elongation in neurons (Okazaki *et al.*, 2000). Together, these findings support the role of GABARAP in the intracellular and membrane trafficking of $GABA_A$ receptors.

Plic-1, a ubiquitin-like protein that is associated with the ubiquitination-degradation machinery (proteasome/ubiquitin-ligase), binds to the $GABA_A$ receptor α and β subunits (Bedford *et al.*, 2001). Plic-1 contains a ubiquitin-like N-terminus that is 33% identical to ubiquitin. It also contains a carboxy-terminal ubiquitin-associated domain that interacts directly with the intracellular loop of the α- and β-subunit containing $GABA_A$ receptors. Functionally, Plic-1 facilitates $GABA_A$ receptor membrane insertion by increasing the half-life of intracellular receptor pools without modifying receptor endocytosis (Bedford *et al.*, 2001). This suggests that this protein plays an important role in the stabilization of cell surface $GABA_A$ receptors by inhibiting their degradation by the proteasomes/ubiquitination. Plic-1 also leads to stabilization of $GABA_A$ receptor subunits in the endoplasmatic reticulum, which increases the production of heteromeric receptors (Saliba RS and Moss SJ, unpublished observations).

There are other factors that affect anterograde transport in the $GABA_A$ receptor trafficking. These include HAP1 (Huntingtin-associated protein-1) (Kittler *et al.*, 2004), GRIF-1 ($GABA_A$ receptor interacting factor-1) (Beck *et al.*, 2002) and GRIP-1 (glutamate receptor interacting protein 1) (Kittler *et al.*, 2004).

HAP1 plays a key role in endocytosis of membrane receptors (Gauthier *et al.*, 2004) and vesicular trafficking as it binds directly to $GABA_A$ receptors, preventing their degradation and thereby enhancing receptor recycling to the plasma membrane (Kittler *et al.*, 2004). Based on its interaction with the microtubule transporter dynactin p150Glued (Engelender *et al.*, 1997; Li *et al.*, 1998) HAP1 might be involved in intracellular trafficking. The microtubule transporter dynactin p150Glued binds to dynein, a microtubule motor that participates in retrograde transport in cells. An interaction between the β subunit of $GABA_A$ receptors and the HAP1 protein is thought to decrease $GABA_A$ receptor lysosomal degradation and to facilitate the recycling of $GABA_A$ receptors back to the cell membrane (Kittler *et al.*, 2004). *GRIF-1* is a member of a new coiled-coil domain family of proteins thought to function as adaptors in the anterograde trafficking of organelles utilizing the kinesin-1 motor proteins to synapses. GRIF-1, which is expressed only in excitable tissues, interacts with the intracellular loop of the β2 $GABA_A$ receptor subunit (Beck *et al.*, 2002; Brickley *et al.*, 2005). These observations support a role for GRIF-1 in protein-vesicle transport in excitable cells in a manner analogous to that of *GRIP-1* (see above).

Palmitoylation or thioacylation of cysteine residues within the γ2 subunit intracellular domain was found to regulate both the formation of $GABA_A$ receptor clusters and total cell surface receptor accumulation (Rathenberg *et al.*, 2004). Mutations of these major four cysteine residues (C359, C368, C375/376, and C380) in γ2 subunit abolished synaptic $GABA_A$ receptor clustering without affecting assembly with receptor α and β subunits. *GODZ* (Golgi-specific DHHC zinc finger protein) is another $GABA_A$ receptor interacting protein which acts as a neuron-specific thioacyltransferase that palmitoylates the intracellular loop of the γ2-subunit containing $GABA_A$ receptor (Keller *et al.*, 2004).

The accumulation of $GABA_A$ receptors at postsynaptic specializations is prerequisite for efficient fast synaptic inhibition. However, how neurons organize this process remains poorly understood. Two new studies analyzed the sites of receptor endo- and exocytosis using electrophysiological (Thomas *et al.*,

2005) or imaging tools (Bogdanov *et al.*, 2006). Imaging with a pHluorin and an α-bungarotoxin-binding site containing $GABA_A$ receptor β3 subunit revealed that extrasynaptic, but not synaptic, $GABA_A$ receptors exhibit rapid rates of clathrin-dependent endocytosis in hippocampal neurons. Extrasynaptic receptors also exhibited preferential colocalization with the AP2 clathrin adaptin. Using pulse chase analysis, newly inserted $GABA_A$ receptors were first detected on the neuronal cell surface within 5 minutes, but did not reach stastically significant levels at synaptic sites until after 30 minutes. Together these results confirm that $GABA_A$ receptor endo- and exocytosis are primarly localized to extrasynaptic membrane sites and that synaptic receptors are directly recruited by lateral diffusion of membrane-bound receptors from this dynamic extrasynaptic $GABA_A$ receptor pool.

CONCLUDING COMMENTS

Molecular studies have revealed a large diversity of $GABA_A$ receptor structure in the brain. This structural diversity allows neurons to assemble a large range of $GABA_A$ receptor subtypes, which have distinct pharmacological and physiological properties. Moreover, it is also apparent that these receptor subtypes are dynamic entities on the neuronal cell surface and that their trafficking itineraries can be subject to dynamic regulation, by specific receptor associated proteins or via the stoichiometry of receptor phosphorylation. Therefore, it will be of major significance to address the relevance of these emerging regulatory mechanisms for the numerous pathologies; which include insomnia, anxiety, premenstrual syndrome, alcoholism, schizophrenia, depression and epilepsy in which altered $GABA_A$ receptor expression has been strongly implicated.

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