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Mechanisms of $GABA_A$ Receptor Assembly and Trafficking

Implications for the Modulation of Inhibitory Neurotransmission

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Abstract

Fast synaptic inhibition in the brain is largely mediated by ionotropic GABA receptors, which can be subdivided into $GABA_A$ and $GABA_C$ receptors based on pharmacological and molecular criteria. $GABA_A$ receptors are important therapeutic targets for a range of sedative, anxiolytic, and hypnotic agents and are implicated in several diseases including epilepsy, anxiety, depression, and substance abuse. In addition, modulating the efficacy of GABAergic neurotransmission may play a key role in neuronal plasticity. Recent studies have begun to reveal that the accumulation of ionotropic $GABA_A$ receptors at synapses is a highly regulated process that is facilitated by receptor-associated proteins and other cell-signaling molecules. This review focuses on recent experimental evidence detailing the mechanisms that control the assembly and transport of functional ionotropic $GABA_A$ receptors to cell surface sites, in addition to their stability at synaptic sites. These regulatory processes will be discussed within the context of the dynamic modulation of synaptic inhibition in the central nervous system (CNS).

Index Entries: Gephyrin; PLIC; GABARAP; assembly; endoplasmic reticulum; ER; targeting; inhibitory; synapse; GABA; ion channel; endocytosis; receptor; dynamin; NSF; AP2; clathrin.

Introduction

γ-Aminobutyric acid (GABA) mediates the majority of inhibitory neurotransmission in the brain. GABA acts at ionotropic $GABA_A$ and $GABA_C$ receptors, which are GABA-gated chloride channels, in addition to metabotropic $GABA_B$ receptors, which are coupled to G-proteins *(1,2)*. In addition to being key sites for synaptic inhibition, $GABA_A$ receptors are important drug targets for anxiolytics, hypnotics and anticonvulsants, including benzodiazepines, barbiturates, neurosteroids and some anesthetics *(3,4)*. Changes in the functional properties of GABA receptors are relevant in a

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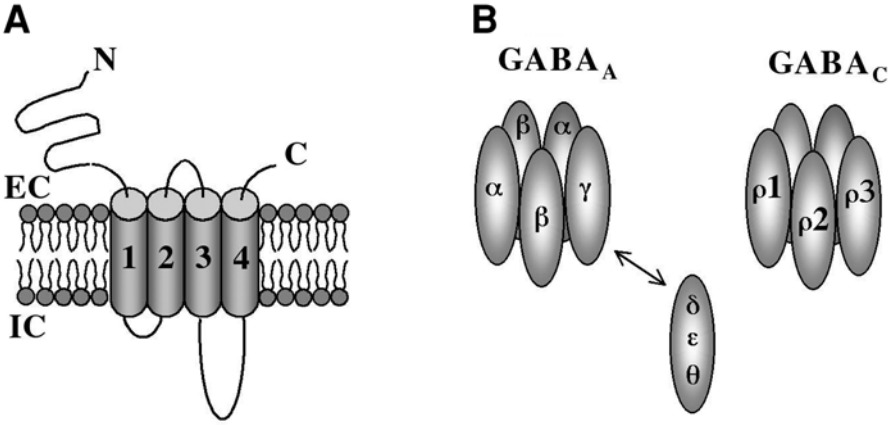

Fig. 1. Structure of ionotropic GABA receptors. **(A)** GABA receptor subunits share a common topology. Each subunit comprises extracellular (EC) amino- and carboxyl-domains and four transmembrane (TM) regions. The major intracellular (IC) loop between TM3 and TM4 is believed to mediate protein–protein interactions. **(B)** $GABA_A$ and $GABA_c$ receptor subtypes have a pentameric structure. Although most neuronal $GABA_A$ receptors are constructed using α, β, and γ subunits in the ratio 2:2:1, the γ subunit can be exchanged for a δ, ε, or θ subunit in some receptor assemblies. $GABA_c$ receptors can form homomeric and heteromeric channels using ρ1–3 subunits. Their stoichiometry is unknown.

number of diseases including epilepsy, depression, anxiety, substance abuse and nociception *(1,2,5–8).*

$GABA_A$ and $GABA_C$ receptors are members of the ligand-gated ion channel superfamily that includes nicotinic acetylcholine, glycine and 5-hydroxytryptamine ($5\text{-}HT_3$) receptors *(9).* Members of this family are pentameric hetero-oligomers, the subunits of which share a conserved structure (*see* Fig.1A) consisting of a large extracellular amino terminus, four transmembrane (TM) domains and a large intracellular loop between TM domains III and IV *(3,9).* $GABA_A$ receptors can be assembled from 16 distinct subunits encoded by separate genes that have been classified by sequence identity into seven subunit classes: α(1–6), β(1–3), γ(1–3), δ, ε, π and θ (*see* Fig.1B) *(2–4,10,11).* The alternative splicing of some subunit mRNAs generates additional subunit diversity *(3,4).* A distinct class of homologous subunits ρ1–3 have been identified, which when expressed as hetero- or homo-oligomers produce GABA-gated channels with unique pharmacology and channel properties similar to those of native receptors expressed primarily in the retina—these have been classified as $GABA_C$ receptors (*see* Fig.1B) *(12).* This review focuses on recent advances in our understanding of the mechanisms important for the assembly and cell-surface stability of ionotropic $GABA_A$ receptors, and the implications of these processes in the regulation of the efficacy of synaptic inhibition.

Subunit Composition of Receptors

In situ hybridization and immunohistochemistry have revealed that the expression of $GABA_A$ receptor subunits is both spatially and developmentally regulated, with individual subunits having distinct but overlapping distributions in brain *(13–16).* The large variety of $GABA_A$ receptor subunits provides the potential for considerable heterogeneity of receptor structure and it has therefore been difficult to identify precisely the subunit com-

position of a single GABA$_A$ receptor subtype in the brain. However, some important conclusions on receptor structure have been made from recombinant receptor expression studies. β1 and β3 subunits can form spontaneously open chloride channels that are inhibited by picrotoxin and activated by pentobarbital, but not GABA *(17–20).* However, there is a general consensus that single α, γ or δ subunits expressed alone do not form GABA-gated channels *(10,11,21–23).* Co-expression of α and β subunits results in the production of GABA-gated channels, which can be blocked by bicuculline and Zn^{2+}, and potentiated by barbiturates *(24–27).* Co-expression of a γ subunit with α and β subunits produces receptors that are potentiated by benzodiazepines (*see* Fig. 1B) *(27a)* but insensitive to inhibition by Zn^{2+} *(27).* Therefore, the majority of GABA$_A$ receptor subtypes in the brain are believed to contain at least 1α, 1β, and 1γ subunit. α1, β2, and γ2 subunits are expressed together in many brain regions, which suggests that they may co-assemble to form an important receptor population *(14).* The prevalent subunit stoichiometry of these receptors is believed to be 2α, 2β, and 1γ subunit *(28).* The importance of this receptor subunit combination was confirmed in studies using transgenic mice lacking either the α1 or β2 subunit, which showed a loss of more than 50% of total GABA$_A$ receptors *(29,30).* Surprisingly, loss of this receptor subtype is not lethal and does not cause seizures *(29,30),* suggesting that in vivo, mechanisms may exist to compensate for the loss of these receptor subunits *(31,32).* However, deletion of either β3 or γ2 subunits produces a lethal phenotype that is evident within hours of birth, suggesting an essential role for both of these GABA$_A$ receptor subunits *(2,32a–c).* GABA$_A$ receptor δ, ε, and θ subunits have a more limited expression-pattern in the brain. It is thought that these subunits can assemble with α and β subunits and substitute for the γ subunit (Fig. 1B) to form GABA$_A$ receptors that are insensitive to benzodiazepines, and that have a higher agonist affinity *(3,10,22,33,34).*

GABA$_A$ Receptor Oligomerisation and Transport to the Cell Surface

From the results described above, it can be concluded that only a subset of GABA$_A$ receptor subunit combinations form functional ion channels, even though there is theoretically a very high number of possible subunit combinations that could assemble into pentameric channels. Therefore, specific regulatory mechanisms must ensure that only appropriate GABA$_A$ receptor subunit complexes can access the cell surface. Evidence from studies of GABA$_A$ receptors as well as other receptors and channels, including nAChRs, GABA$_B$ receptors and NMDA receptors suggests that receptor assembly occurs in the endoplasmic reticulum (ER), which serves as checkpoint to ensure efficient assembly of functional receptor/channel complexes *(1,35–38).* Studies of recombinant GABA$_A$ receptors expressed in heterologous systems composed of α1, α6, β1–2, and γ2L subunits have revealed that access to the cell surface is limited to αβ and αβγ subunit combinations *(19,23,39,40).* Expression studies in human embryonic kidney (HEK) 293 cells have shown that single subunit combinations (α1, β2 and γ2L) and α/γ2S, subunit combinations are retained within the ER *(19,23,40,41).* Moreover, when expressed alone the α1 and β2 subunits are unstable and rapidly degraded *(42).* The retention and degradation of misfolded GABA$_A$ receptor subunits is directed by ER chaperone molecules *(43)* as GABA$_A$ receptor subunits associate with both Heavy Chain Binding protein (BiP) and Calnexin *(23,42).* Surprisingly, differential cell-surface trafficking of γ2L and γ2S subunit splice variants has also been demonstrated. The two subunits differ by the presence of only 8 amino acids within their major intracellular domain *(44,45).* Whereas the γ2L subunit is retained within the ER when expressed alone, the γ2S subunit can access the cell surface as a non-functional monomer where it is subsequently removed rapidly by endocytosis *(46).* However, co-expression of the γ2S subunit with either the α1 or β2 subunit

results in the retention of γ2S in the ER, suggesting that, in vivo the γ2S subunit is unlikely to occur as a monomer *(41).*

Importantly, similar mechanisms of ER retention of single subunit combinations occur in neurons. Infection of cultured superior cervical ganglion (SCG) neurons with Semliki Forest viruses expressing $GABA_A$ receptor subunits revealed that α1 or β2 subunits expressed alone are retained intracellularly, whereas co-expression of α1 and β2 subunits results in cell-surface receptor targeting in neuronal cell bodies and dendrites *(42).* Similarly, microinjection of cultured hippocampal neurons with recombinant $GABA_A$ receptor subunits revealed that the γ2L subunit is retained in the ER when expressed alone, and can only be targeted to the cell surface and synapses when co-expressed with α1 and β2 subunits *(47).* This provides good evidence that assembly of neuronal receptors occurs in the ER, and that only a subset of receptor subunit combinations are able to access the cell surface.

The mechanisms of ER-retention of receptor subunits are not fully understood. It is possible that some subunits can only fold correctly when fully assembled and therefore in the absence of the necessary oligomerization partners, they are bound by chaperones and targeted for degradation. Alternatively, the hetero-oligomerization of receptor subunits into functional receptor complexes may mask ER-retention signals that retain unassembled subunits or subunit combinations that are not fully able to assemble into functional receptors. This has been shown for both $GABA_B$ receptors (reviewed in ref. *1*) and NMDA receptor subunits *(38).* Evidence supporting the requirement of $GABA_A$ receptor oligomerization for ER exit comes from studies of the trafficking of $GABA_A$ receptor β2 and β3 subunit chimeras. Whereas β2 subunits are retained in the ER when expressed alone, β3 subunits are able to homo-oligomerize, exit the ER and access the cell surface *(19,20,23).* However, exchanging the β2 subunit extracellular domain with that of β3 confers on this chimera the ability to homo-oligomerize and exit the ER *(48).*

$GABA_A$ receptor subunit ER retention may also serve to regulate the availability of particular subunits for assembly and subsequent insertion of new $GABA_A$ receptors into synapses. In cultured hippocampal neurons, ER-retained, exogenously expressed γ2 subunits are immunostained in the cell soma and dendritic processes *(47).* The presence of ER and Golgi-associated proteins in dendrites suggests that synapses are equipped with the necessary machinery required for the synthesis and assembly of correctly folded complex proteins *(49).* The ER retention of specific $GABA_A$ receptor subunits in dendrites could provide a mechanism for the regulated transport and targeting of receptors to synapses, and allow for a rapid and localized modification of inhibitory synapse receptor number. A similar mechanism has been suggested for NMDA receptor NR1 subunits and glycine receptors *(38,49,50).*

Specification of $GABA_A$ Receptor Subunit–Subunit Interactions

For most $GABA_A$ receptor subunits, assembly into hetero-oligomeric complexes facilitates access to the cell surface. Importantly, specific amino acid sequences present in subunit N-termini have been found to control $GABA_A$ receptor subunit oligomerization *(40,48,51–54).* Using a chimeric approach, four amino acids in the N-terminal domain of the β3 subunit have been identified that mediate homo-oligomerization and functional cell-surface expression of this subunit compared to β2, which is retained within the ER *(48).* Substitution of these four amino acids into the β2 subunit is sufficient to enable the β2 subunit to homo-oligomerize and exit the ER. Interestingly, these residues are not critical for formation of α1β2 or α1β3 hetero-oligomers, but are important for the oligomerization of β subunits with γ subunits. Studies have also identified a domain in α1 (amino acids 58–67), conserved in all α subunit isoforms that is essential for

oligomerization of α1 subunits with β3 subunits, but not the γ2 subunit and therefore plays a critical role in assembly of αβ- and αβγ-containing receptors *(40)*. Significantly, residue F64 from this domain contributes to the GABA-binding site that is thought to be located at the α1–β3 subunit interface *(55)*. Furthermore, Q67 and W69 from this domain have been identified to play a particularly important role in mediating GABA$_A$ receptor assembly *(40,54)*. W94 has also been implicated as essential for GABA$_A$ receptor assembly *(54)*. Interestingly, W69 and W94 are common to all ligand-gated ion channel subunits, whereas Q67 is conserved in GABA$_A$, 5-HT$_3$, nAChR α, and glycine receptor subunits. This suggests that these conserved residues may play a key role in the assembly of all ligand-gated ion channels.

With an approach based on truncated GABA$_A$ receptor N-terminal domains, Klausberger and colleagues have identified other amino-acid sequences important for receptor assembly *(51–53)*. Using C-terminal truncated γ2 subunits as well as mutated and chimeric fragments, amino acid sequences γ2 (91–104) and γ2 (83–90) were identified to mediate assembly of the γ2 subunit with α1 and β3 subunits respectively *(51)*. With a similar approach, a reciprocal γ2 subunit-binding domain in the α1 subunit (α1-amino acids 80–100) was identified *(53)*. Importantly, this region was found to affect assembly with γ2 but not β3, and to interact directly with the 91–104 amino acid region of γ2 *(53)*. From the above results, Klausberger et al. suggest a model of GABA$_A$-receptor assembly where α1 subunits contain at least three distinct subunit-binding sites. Two binding sites on opposite faces of the N-terminal domain can mediate assembly with β3 subunits, and on one face there is an additional binding site for γ2 subunits. This allows the α1 subunit to bind either to two β3 subunits, or to one β3 and one γ2 subunit. Furthermore, they suggest that the single α1–γ2 inter-subunit contact site controls the subunit assembly and stoichiometry of GABA$_A$ receptors *(53)*.

Functional Consequences of GABA$_A$ Receptor Subunit Heterogeneity

The significance of the structural heterogeneity of GABA$_A$ receptors remains unresolved but may be related to the production and subcellular-targeting of receptors with different functional properties. The pharmacological and physiological properties of GABA$_A$ receptors depend on their subunit composition *(3,4,56,57)*. In addition, there is evidence for the differential subcellular targeting of different GABA$_A$ receptor complexes *(19,58–62)*. This suggests that the targeting of receptors with specific properties to defined regions may be necessary for the fine-tuning of the integrative properties of a particular neuron. Studies in both polarized epithelial cells and neurons have revealed that different GABA$_A$ receptor subunit combinations can be sorted to different subcellular domains. In polarized epithelial cells, the subcellular localization of heterologously expressed GABA$_A$ receptors containing α1 and β1, and β2 or β3 subunits is determined by the identity of the β-subunit. Whereas α1/β2 and α1/β3 subunit combinations are targeted to the basolateral domain of these cells, α/β1 complexes exhibit a non-polarized distribution *(19)*. The differential subcellular trafficking of receptor subunit combinations has also been demonstrated in vivo. Studies using immunogold electron microscopy have identified the differential synaptic localization of two major GABA$_A$ receptor α subunits on hippocampal pyramidal cells. Whereas α1 subunits are localized to the majority of GABAergic synapses, α2 subunits can only be detected in a subset of somatodendritic synapses and are mainly enriched at synapses on the axon initial segment *(58)*. Immunofluorescence studies have demonstrated differential targeting of α2- and α5-containing GABA$_A$ receptors in hippocampal pyramidal cells and olfactory bulb granule cells, and non-overlapping distributions of α1, α2, and α3 subunits in retinal α-ganglion cells *(59,60)*.

In cerebellar granule cells, studies focusing on the subcellular distribution of α1, α6, β2, β3, γ2

and δ subunits have revealed the differential targeting of specific subunit combinations *(61,62).* Immunogold localization indicates that receptors containing α1, α6, β2/3 and γ2 subunits are concentrated at GABAergic Golgi synapses, and found at lower levels at extrasynaptic sites. In contrast, the δ subunit is present only at dendritic and somatic extrasynaptic sites.

Accumulating evidence suggests that different subunit combinations may underlie distinct forms of neuronal inhibition important for controlling cell excitability. In both hippocampal pyramidal cells and cerebellar granule neurons, synaptic and extrasynaptic receptors appear to have different functional properties *(63,64).* For example, the relatively slow deactivation kinetics of extrasynaptic $GABA_A$ receptors on CA1 pyramidal neurons may be specialized for mediating tonic inhibition *(64).* The role of different receptor subtypes in mediating various types of GABA-mediated inhibition has been characterized in the cerebellum. Here, extrasynaptic $GABA_A$ receptors set the resting conductance of the postsynaptic cell via tonic activation by ambient GABA or GABA-spillover from the synaptic cleft *(33,63,65–67).* The extrasynaptic α6 and δ subunit-containing receptors in cerebellar granule cells are well suited for this role, as they have a high affinity for GABA and do not desensitize *(33,34).* Evidence in support of α6/δ subunit-containing receptors mediating tonic- and spillover-inhibition comes from studies of α6 knockout mice (which lack both α6 and δ subunits in the cerebellum *[68]*), where the tonic conductance is absent in cerebellar granule cells from these animals *(31)* and from pharmacological studies *(67,69).* In agreement with this, knockout of the α1 subunit does not affect the tonic conductance in cerebellar granule cells *(29).* This tonic- and spillover-inhibition of cerebellar granule cells appears to play a major role in controlling information flow through the cerebellar cortex *(69).* The importance of tonic-inhibition (mediated by extrasynaptic α6/δ-containing receptors) in cerebellar function is further highlighted by the fact that in α6 knockout animals, the response of granule cells to excitatory input remains unaltered due to the increase in a 'leak' conductance with properties of the TASK-1 two-pore-domain K^+ channel *(31).* Therefore, loss of $GABA_A$ receptor mediated tonic-inhibition triggers a form of homeostatic plasticity, leading to a change in magnitude of a voltage-independent K^+ conductance to maintain normal behavior *(31).* These results provide compelling evidence for distinct roles of synaptic versus extrasynaptic $GABA_A$ receptors containing different receptor-subunit combinations in GABA-mediated control of cell excitability.

Control of $GABA_A$ Receptor Cell-Surface Stability

Given the central role that $GABA_A$ receptors play as mediators of inhibitory neurotransmission, it is important to understand how receptor function is regulated at the neuronal surface. Modulation of the functional properties and membrane stability of $GABA_A$ receptors is a major determinant of inhibitory synaptic strength, and therefore may be involved in synaptic plasticity. Modifications governing the efficacy of neurotransmission occur both pre- and postsynaptically *(70,71).* However, if the receptors on the postsynaptic membrane have a high open probability, then increasing their number may provide an effective means of increasing synaptic strength, as they are likely to be saturated by released transmitter *(72–76).* Several studies have provided evidence that the number of postsynaptic $GABA_A$ receptors underlies the efficacy of inhibitory synapses, and support the idea that $GABA_A$ receptors cycle between synaptic sites and intracellular compartments. In cerebellar stellate cells, differences in the amplitude of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) are caused by variability in the number of postsynaptic receptors *(77).* Furthermore, potentiation at hippocampal inhibitory synapses occurs in response to an increase in the number of postsynaptic $GABA_A$ receptors following kindling-

induced epilepsy *(78,79).* A study showing an insulin-induced increase in the number of postsynaptic $GABA_A$ receptors has provided evidence that rapid (tens of minutes) redistribution of receptors can occur from intracellular compartments to the cell surface *(80).* Furthermore, it is suggested that this translocation of $GABA_A$ receptors to the plasma membrane underlies the enhanced amplitude of mIPSCs seen in response to insulin in cultured hippocampal neurons *(80).* Conversely, a reduction in the number of postsynaptic $GABA_A$ receptors is proposed to account for the decreased amplitude of mIPSCs in cultured hippocampal neurons in response to brain-derived neurotrophic factor (BDNF) *(81).* Blocking postsynaptic receptor removal via clathrin-dependent endocytosis leads to an increase in the amplitude of mIPSCs, which may be accounted for by a subsequent accumulation of postsynaptic $GABA_A$ receptors *(82).* $GABA_A$ receptors undergo constitutive endocytosis (*see* Fig. 2) in heterologous cells and cultured neurons *(41,46,82).* In HEK 293 cells, endocytosis of $GABA_A$ receptors is clathrin-dependent *(41,46,82).* Furthermore, receptors composed of α and β subunits appear to be internalized to peripheral endosomes, whereas αβγ-containing receptors are targeted to late peri-nuclear endosomes *(46).* In addition, activation of protein kinase C (PKC) leads to down-regulation of $GABA_A$ receptors by inhibiting the recycling of receptors from late endosomes to the cell surface in recombinant systems *(46,47).* This occurs by a mechanism believed to be independent of direct receptor phosphorylation *(46).*

Recruitment into clathrin-coated pits is a key mechanism involved in the endocytosis of many neuronal receptors, including opioid receptors *(83),* the β-adrenergic receptor *(84),* and ionotropic glutamate receptors *(85–88).* The translocation of such membrane proteins involves adaptor proteins, which act to target the receptor to specific components of the endocytic machinery, such as clathrin, the GTPase dynamin, and its binding partner amphiphysin *(89).* Several studies now suggest that internalization of $GABA_A$ receptors in neurons is clathrin-dependent (Fig. 2). $GABA_A$ receptors are present on clathrin-coated vesicles in brain *(90–92)* and co-localize with the adaptin AP2 complex in cultured hippocampal neurons *(82).* In addition, $GABA_A$ receptor α and β subunits can associate with the AP2 adaptor complex. Therefore, the recruitment of $GABA_A$ receptors to clathrin-coated pits may involve this adaptor protein *(82).* Exposure of cultured hippocampal neurons to a peptide that disrupts the interaction between dynamin and amphiphysin, and consequently blocks dynamin-dependent endocytosis, leads to an increase in the function of synaptic $GABA_A$ receptors *(82).* Together, these studies suggest that regulation of clathrin-dependent endocytosis may play a pivotal role in determining the number of $GABA_A$ receptors on the postsynaptic membrane. The precise cues that control $GABA_A$-receptor internalization remain to be identified, but it is evident that internalization is mediated by both agonist-dependent and independent mechanisms *(82,90,91,93).* The role of both $GABA_A$-receptor agonists and allosteric modulators in internalization is an area of active interest, as this process may provide a mechanism for the development of tolerance to both benzodiazepines and barbiturates, which severely limits their clinical use.

The Role of Associated Proteins in $GABA_A$-Receptor Trafficking

The selective targeting and clustering of $GABA_A$ receptors at synaptic sites is essential for efficient inhibitory synaptic transmission *(94),* and even a partial decrease in $GABA_A$-receptor clustering results in significant behavioral dysfunction *(95).* The multifunctional protein gephyrin is essential for $GABA_A$-receptor clustering and appears to act as a key organizing/scaffolding molecule at inhibitory synapses (reviewed in refs. *2,96,97*). Gephyrin was first identified as a protein that mediates the synaptic-clustering of glycine receptors via a direct interaction with the intracellular domain

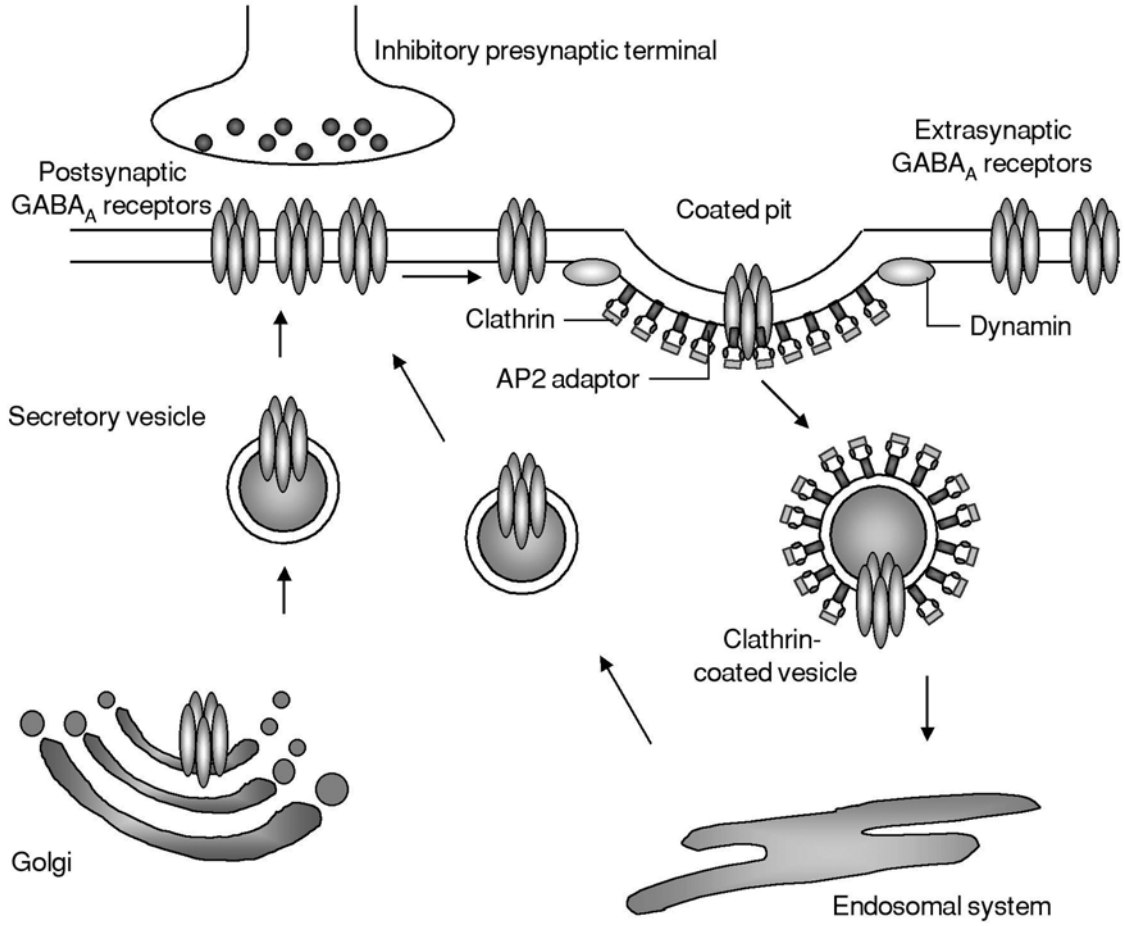

Fig. 2. Trafficking and membrane targeting of $GABA_A$ receptors at inhibitory synapses. This figure shows a subset of proteins implicated in $GABA_A$ receptor endocytosis. Constitutive endocytosis of postsynaptic $GABA_A$ receptors occurs via a clathrin-mediated dynamin-dependent pathway. Recruitment of these receptors to clathrin-coated pits involves an interaction with the AP2 adaptor. Clathrin-coated vesicles subsequently target the receptors to endosomes where they are either targeted for degradation or recycled back to the plasma membrane. $GABA_A$ receptors are also synthesized *de novo* and targeted to the cell surface. It is presently not known if recycled and *de novo* synthesized receptors are directly inserted at synaptic membranes, or if they are inserted into extrasynaptic membranes and then sequestered at synapses.

of the receptor β subunit. In addition, gephyrin co-localizes with $GABA_A$-receptor clusters in many brain regions and depletion of gephyrin using antisense oligonucleotides results in a disruption of $GABA_A$-receptor clusters *(94).* Furthermore, in a gephyrin knockout mouse there is an almost complete loss of $GABA_A$-receptor clusters containing the α2 and γ2 subunits *(98).* However, to date, a direct interaction between $GABA_A$ receptors and gephyrin has not been demonstrated, suggesting that these two proteins may be linked by an unidentified factor(s). It is evident that the $GABA_A$ receptor γ2 subunit plays a critical role in controlling the targeting to and/or clustering of $GABA_A$ receptors at inhibitory synapses. Deletion of the receptor γ2 subunit produces a 70% decrease in receptor number at inhibitory synapses, and

a parallel loss of gephyrin staining *(94).* Evidence for a role of presynaptic factors has also been provided by an elegant study that used the culture of motor neurons alone or co-cultured with either glycine or GABA interneurons *(98a).* Gephyrin was found to accumulate at synapses regardless of the identity of the transmitter released (for example, GABA, glycine or acetylcholine), whereas GABAergic innervation resulted in the synaptic accumulation of $GABA_A$ receptors but not glycine receptors. Only upon innervation from spinal interneurons that release both GABA and glycine was synaptic glycine receptor accumulation observed. Under these conditions it appears that gephyrin, $GABA_A$ receptors (detected with an antibody to the $GABA_A$ receptor β3 subunit) and glycine receptors can be detected at the same synapse on motorneurons apposed to GABAergic terminals and suggests that at this synapse these two receptor types can intermingle using gephyrin. Interestingly, most brain areas that express glycine receptor α subunits mainly express $GABA_A$ receptor β3 subunits, and not β2 *(98b),* suggesting that $GABA_A$ receptors containing the β3 subunit may favor mixing with glycine receptors.

To further identify proteins that are important for controlling $GABA_A$-receptor membrane trafficking, subunit-binding partners have been isolated using yeast-two hybrid screening *(99–101)* (*see* Fig. 3). The first molecule to be discovered by this method was the $GABA_A$-receptor-associated protein (GABARAP). This 17-kDa polypeptide was isolated using the intracellular domain of the $GABA_A$ receptor γ2 subunit, and has been shown to associate with $GABA_A$ receptors in vivo via a selective interaction with γ subunits *(99,102).* GABARAP is a member of a novel family of proteins that are involved in vesicular transport and membrane traffic processes *(103–105).* These include the mammalian homologs, light chain 3 (LC3), and Golgi-associated ATPase enhancer (GATE-16), in addition to the yeast ortholog Apg8p/Aut7p. Apg8/Aut7p and LC3 are involved in autophagocytosis whereas GATE-16 plays a role in Golgi transport and can interact with the trafficking protein *N*-ethylmaleimide-sensitive factor (NSF) *(104,106).*

The role of GABARAP in $GABA_A$-receptor function is unclear. GABARAP can bind both soluble and polymerized forms of tubulin, in vitro, and microtubules, in vivo *(99,107,108),* which suggests that it may anchor $GABA_A$ receptors to the cytoskeleton at synapses, and promote receptor clustering. In agreement with this, overexpressing GABARAP with $GABA_A$ receptors in a mammalian cell-line results in increased numbers of clustered receptors that have altered kinetics, including faster deactivation and slower desensitization *(109).* The identification of an association between GABARAP and gephyrin also suggests that GABARAP may serve as a linker-molecule between $GABA_A$ receptors and gephyrin *(96,110,111).* Further insight into the possible cellular function of GABARAP has been achieved following determination of the crystal structure and structure-function studies *(108,112,113).* GABARAP comprises an N-terminal tubulin-binding region, which is important for tubulin polymerization, and a ubiquitin-like C-terminal domain, which binds the intracellular loop of $GABA_A$-receptor subunits and may interact with other GABARAP-binding partners *(107–109).* This region of GABARAP is able to bind an 11 mer minimal binding peptide from the γ2 subunit intracellular loop *(99,102,107)* with an affinity in the low micromolar range ($K_D = 1.3 \times 10^{-6}$) *(108).* From their studies, Coyle and colleagues suggest that GABARAP can exist in two states: a closed monomeric form stabilized by intra-molecular interactions, and an open state which can self-associate and oligomerize *(108).* In this head-to-tail arrangement, GABARAP is believed to retain functional properties *(108)* and it is possible that interactions between GABARAP and cellular proteins may induce this conformational change to promote tubulin polymerization and clustering of $GABA_A$ receptors *(108).*

Surprisingly, low levels of GABARAP are found at GABAergic synapses and do not co-localize significantly with gephyrin. This suggests that GABARAP is not a core component

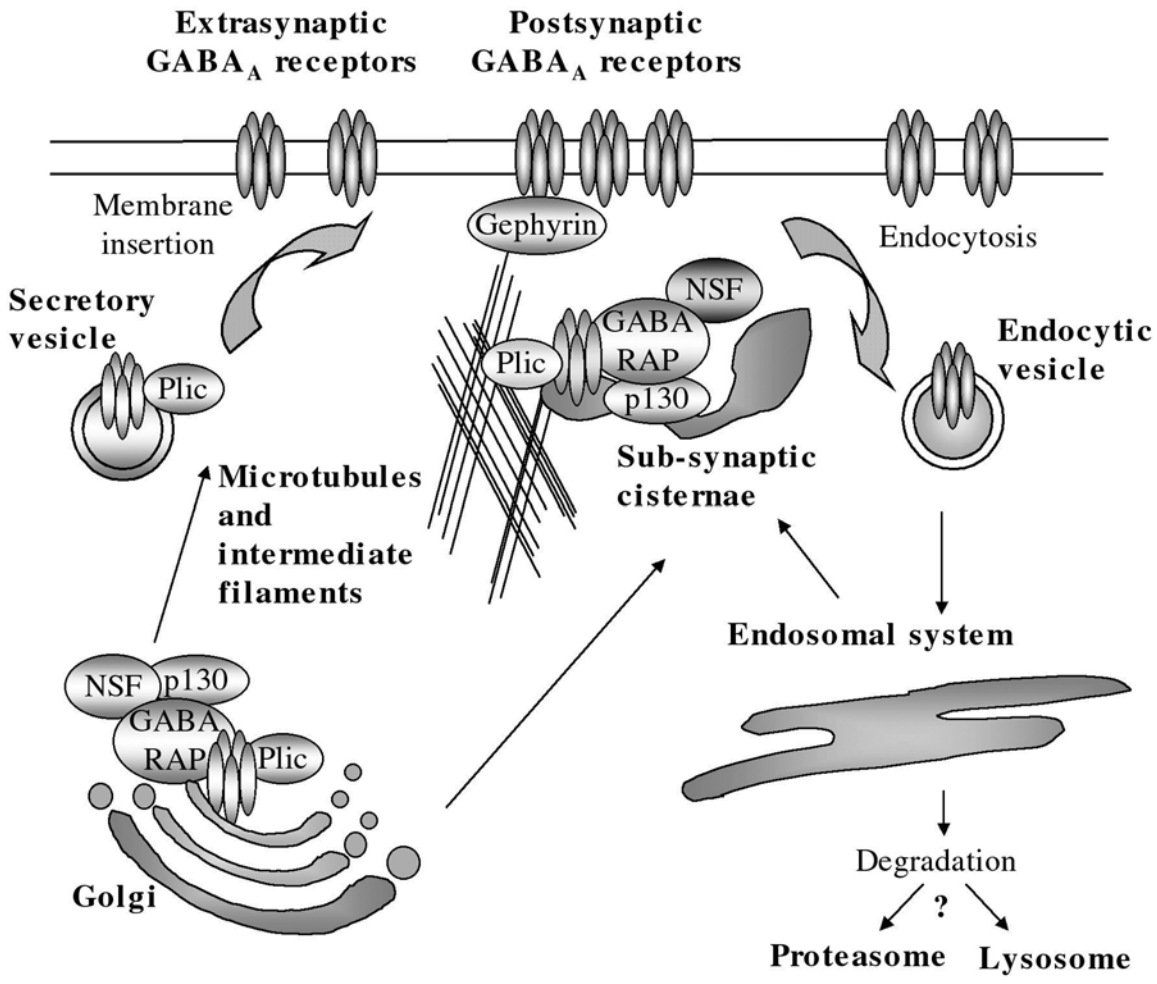

Fig. 3. Proteins associated with the trafficking of $GABA_A$ receptors. $GABA_A$ receptors are clustered at synapses by the inhibitory synaptic scaffold gephyrin through an, as yet unknown, mechanism. The trafficking of *de novo* synthesized and endocytosed $GABA_A$ receptors is facilitated by receptor-associated proteins. Plic proteins increase receptor half-life and cell-surface number. Similarly, GABARAP may facilitate $GABA_A$-receptor trafficking via association with the proteins NSF and p130. The interaction of gephyrin with collybistin and profillin has also been demonstrated *(101a,101b)* but is not shown in this figure.

of the inhibitory synaptic scaffold and is unlikely to act as a synaptic-bridging molecule between $GABA_A$ receptors, the cytoskeleton, and gephyrin *(105,110)*. GABARAP is localized predominantly to intracellular membranes and detailed immuno-electron microscopy studies revealed that it is enriched at the edges of Golgi stacks and in a sub-synaptic tubulovesicular compartment *(105)*. Furthermore, GABARAP associates directly with proteins involved in vesicular transport, including NSF. Complexes of GABARAP and NSF have been identified in neurons where they co-localize on intracellular membranes *(105)*. These results support a role for GABARAP in the intracellular transport of $GABA_A$ receptors. This may be reconciled with the proposed clustering-function for GABARAP as suggested by Chen

and colleagues *(109)* if GABARAP increases $GABA_A$-receptor clustering by enhancing cell-surface receptor targeting and plasma-membrane accumulation. Therefore, GABARAP may regulate rates of receptor-membrane insertion or endocytosis by functioning as an adaptor molecule that links cargo molecules such as $GABA_A$-receptor-containing vesicles to the cytoskeleton. It has recently been revealed, in an elegant study by Kanematsu et al. (2002) *(114)*, that the binding of GABARAP to the $GABA_A$ receptor γ2 subunit is regulated by another binding protein: catalytically inactive phospholipase C (p130). A yeast-two hybrid screen with p130 identified an association with GABARAP and it was also found to compete with GABARAP for binding to the γ2 subunit *(114)*. This interaction is likely to be of major significance, as genetic deletion of p130 produces $GABA_A$ receptors that have altered responses to both Zn^{2+} and benzodiazepines. In addition, diazepam has significantly reduced sedative and anxiolytic effects on these p130 knockout mice, further confirming an altered function of $GABA_A$ receptors containing the γ2 subunit. Together, these results suggest a role for p130 and GABARAP in the assembly and/or membrane transport of $GABA_A$ receptors.

The ubiquitin-like protein Plic-1 *(115)* (Proteins that *L*ink *I*AP with the Cytoskeleton) has also been shown to bind $GABA_A$ receptors in vivo *(101,116)*. Plic proteins interact with the ubiquitin ligase E6-AP in a complex with proteosomes, and are believed to block the degradation of ubiquitinated substrates to increase their half-life *(117,118)*. Similarly, binding of Plic-1 to $GABA_A$ receptors appears to be important for increasing the half-life of these receptors. Plic-1 has a molecular weight of 67 kDa and contains an ubiquitin-like amino terminus and a C-terminal ubiqutin-associated domain (UBA). Plic-1 interacts with the major intracellular loop of $GABA_A$ receptor α and β subunits via its UBA domain, suggesting that it can interact with most $GABA_A$-receptor subtypes *(101)*. Co-expression studies of $GABA_A$ receptors and Plic-1 have revealed that this protein acts to increase receptor half-life and cell-surface expression without modifying receptor endocytosis *(101)*. This suggests a key role for Plic-1 in stabilizing internal receptor populations for insertion into the plasma membrane. Plic-1 immunoreactivity could be detected co-localized with the receptor at inhibitory synapses, and in addition was found localized to subsynaptic compartments and at the edge of the Golgi apparatus *(101)*—in keeping with a role for this protein in $GABA_A$-receptor stabilization and membrane insertion.

It is interesting to note that Plic-1 and GABARAP appear to have an overlapping subcellular distribution. Both Plic-1 and GABARAP can be detected on intracellular membranes including the edges of the Golgi apparatus, and also in a tubulo-vesicular compartment just below the synapse (subsynaptic cisternae) *(101,105)*. This suggests that Plic-1 and GABARAP may have complementary roles in regulating the trafficking and membrane insertion of $GABA_A$ receptors—particularly in light of the complementary subunit specificity of the two proteins (i.e., Plic-1 binds α and β subunits; GABARAP binds γ subunits). Since both Plic-1 and GABARAP can be detected on subsynaptic cisternae (and in coated pits in the case of Plic-1), they may act in concert to sequester a pool of recycled receptors and target them for membrane reinsertion. In addition, the presence of these two proteins on the Golgi suggests that they may also play a role in the transport/trafficking of *de novo* synthesized receptors, perhaps by targeting newly formed receptors to the same subsynaptic compartment for regulated insertion into the postsynaptic domain.

Concluding Remarks and Future Directions

LTP and LTD of GABAergic synapses has been detected in a number of brain regions *(119–121)*. These changes in the efficacy of $GABA_A$-receptor-mediated control of nerve-cell excitability are likely to have an important

effect on the integrative properties and input–output relationship of a neuron. The modulation of $GABA_A$-receptor-mediated inhibition therefore has important implications for modulating neuronal network activity and information processing in the brain. For example, LTD of GABAergic inhibition has recently been shown to underlie the increased excitability of CA1 neurons associated with LTP *(122).* It will be important to determine if the regulation of $GABA_A$ receptor membrane trafficking underlies some of these previously reported forms of GABAergic plasticity, as has been reported for LTP and LTD of excitatory receptors *(86,123).* Similarly, the role of receptor-associated 'trafficking' proteins such as Plic-1, AP2, and GABARAP in the regulation of inhibitory synaptic strength needs to be further determined.

Receptor trafficking and endocytosis may also have significant relevance to the development of tolerance and dependence to allosteric modulators of $GABA_A$ receptors, such as benzodiazepines and ethanol *(5,8,124).* Although benzodiazepines are widely prescribed for the treatment of anxiety and sleep disorders, development of significant tolerance and dependence/addiction to these drugs limits their use *(8).* Although the mechanisms that produce tolerance to benzodiazepines are unknown, there is evidence to suggest that receptor endocytosis is implicated in this process *(92,97).* Similarly, the trafficking of $GABA_A$ receptors may be implicated in the development of both acute and chronic tolerance to ethanol *(5).* A complete understanding of the mechanisms of $GABA_A$ receptor assembly and trafficking, and the role these processes play in regulating GABAergic control of cell-excitability, will be important for understanding the molecular mechanisms that underlie neuronal plasticity and addiction.

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