THE DYNAMIC MODULATION OF GABA\textsubscript{A} RECEPTOR TRAFFICKING AND ITS ROLE IN REGULATING THE PLASTICITY OF INHIBITORY SYNAPSES

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Inhibition in the adult mammalian central nervous system (CNS) is mediated by \(\gamma\)-aminobutyric acid (GABA). It is estimated that at least one-third of all CNS neurons utilize GABA as their primary neurotransmitter. Most of these GABAergic neurons are interneurons and therefore are uniquely able to alter the excitability of local circuits within a given brain region. GABA exerts its powerful inhibitory influence by acting on two distinct classes of receptors based on their electrophysiological and pharmacological properties. Ionotropic GABA\textsubscript{A} receptors are fast-acting ligand-gated chloride channels (69), while metabotropic GABA\textsubscript{B} receptors are coupled indirectly via G-proteins to either calcium or potassium channels to produce slow and prolonged inhibitory responses (10). GABA\textsubscript{A}Rs are clinically relevant drug targets for anticonvulsant, anxiolytic, and sedative-hypnotic agents including benzodiazepines, barbiturates, neurosteroids, and some general anesthetics. GABA\textsubscript{A}Rs are heteropentameric ligand-gated ion channels that are found concentrated at inhibitory postsynaptic sites where they mediate phasic inhibition and at extrasynaptic sites where they mediate tonic inhibition. The efficacy of inhibition and thus neuronal excitability is critically dependent on the accumulation of specific GABA\textsubscript{A}R subtypes at inhibitory synapses. Here we evaluate how neurons control the number of GABA\textsubscript{A}Rs on the neuronal plasma membrane together with their selective stabilization at synaptic sites. We then go on to examine the impact that these processes have on the strength of synaptic inhibition and behavior.

I. INTRODUCTION

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The classic GABA\textsubscript{A}R-mediated hyperpolarization of the membrane potential is attributed to the direct activation of an integral ion channel and the resultant influx of chloride along its electrochemical gradient. The concentration-response curve exhibits positive cooperativity consistent with the presence of at least two GABA binding sites on the receptor complex (5). Following brief exposure to high (millimolar) concentrations of GABA (released from presynaptic vesicles), activation of GABA\textsubscript{A}Rs located at synaptic sites transiently moves the membrane potential away from the spike threshold required for action potential generation underlying what is known as “phasic” inhibition. In contrast, low (submicromolar) concentrations of ambient GABA in the extracellular space can persistently activate spatially and temporally less-restricted extrasynaptic receptors to generate a persistent or “tonic” inhibitory state (29). Given the critical role of GABA\textsubscript{A}Rs in controlling neuronal excitability and animal behavior, it is of fundamental im-
Importance to understand the mechanisms used by neurons to regulate their function.

Emerging evidence indicates that activity-dependent changes in the number of postsynaptic GABAARs represent one of the most powerful mechanisms underlying the functional plasticity of GABAergic synapses. Moreover, deficits in the functional expression of GABAARs have been implicated in the pathogenesis of a wide range of neurological and psychiatric diseases including epilepsy, anxiety, depression, schizophrenia, and substance abuse. A significant amount of research has thus focused on detailing the cellular and molecular mechanisms that regulate the stability of GABAARs on the cell surface and their accumulation at the inhibitory postsynapse.

II. GABA<sub>A</sub> RECEPTOR STRUCTURE

GABA<sub>A</sub>Rs belong to the superfamily of Cys-loop ligand-gated ion channels that comprises nicotinic acetylcholine receptors (nAChRs), strychnine-sensitive glycine receptors, and 5-hydroxytryptamine type-3 receptors (5-HT<sub>3</sub>Rs). Members of this family are heteropentameric glycoproteins composed of homologous subunits that specifically recognize one another and assemble around an intrinsic ion channel (FIGURE 1), which in the case of the GABA<sub>A</sub>R is permeable to chloride and, to a lesser extent, bicarbonate anions (81). Each subunit is predicted to share a common membrane topology encompassing a large extracellular NH<sub>2</sub>-terminal ligand-binding domain and four transmembrane (TM) 1–4 domains. A major cytoplasmic loop lies between TM3 and TM4 and is the most divergent part of the sequence among the GABA<sub>A</sub>R subfamily. This intracellular domain (ICD) is the target for a number of posttranslational modifications that can directly affect channel function and/or mediate the interaction with cytosolic proteins important for regulating receptor localization and/or membrane trafficking.

Comparative modeling of the GABA<sub>A</sub>R based on the 4-Å resolution model of the *Torpedo* nACh receptor for the first time provided an insight into the three-dimensional organization of this ligand-gated ion channel (27). The extracellular domain of each receptor subunit comprises a variable NH<sub>2</sub>-terminal domain and two β-folded sheets that form a twisted sandwich connected by a signature disulfide bridge. Each subunit has a principal (+) and a complementary (−) side. The GABA binding sites are located in solvent-accessible pockets at the two β+α− subunit interfaces and that of benzodiazepines at the α+γ− interface (26). The TM
domain of the receptor is made up of four loosely packed helical bundles resulting in a considerable amount of solvent-accessible space within subunits and at the subunit interfaces. It has been proposed that the intersubunit pockets form a continuous groove with their extracellular counterparts, suggesting that they may not only play a role in the conformational mobility of the receptor but also provide putative drug-binding sites.

The subunit interfaces contain a number of amino acid residues that have been shown to be of key importance in the binding and/or efficacy of a number of modulatory drugs. For example, S270 in TM2 and A291 in TM3 in the α1 subunit are of importance for the actions of volatile anesthetics (57). Similarly, an aspartic acid residue (R265) in the β2 and β3 subunit isoforms is believed to account for the subtype-selectivity of etomidate (8). Photoaffinity labeling has also revealed that Met-236 in the α subunit (and/or the homologous methionines in α2, 3, 5), in addition to Met-286 in the β3 subunit, also participate in etomidate’s action at GABA_A Rs (50a). In addition, a single glutamine residue with the α subunit isoforms (Q241) is critical for steroid modulation of GABA_A Rs (34). Future experiments leading to the improvement in the accuracy of the models will not only continue to provide a new perspective on existing data but also pave the way for structure-based drug design.

III. SUBUNIT COMPOSITION OF SYNAPTIC AND EXTRASYNAPTIC GABA_A RECEPTORS

To date, 21 GABA_A subunits have been cloned and sequenced from the mammalian CNS. These have been divided into eight classes on the basis of sequence identity (87): α1(1–6), β1(1–3), γ1(1–3), δ, ε, π, θ, and ρ1(1–3) [FIGURE 1]. Subunit isoforms within a single class share ~70% sequence identity, but between classes this falls to 30–40%. Moreover, alternatively spliced variants of several of these subunits have been reported, generating further subunit diversity and the potential for extensive molecular heterogeneity (70). For example, the γ2 subunit exists in short (γ2S) and long (γ2L) forms, which differ in an eight-amino acid insert in the ICD of the γ2L subunit (47, 86). Despite the plethora of GABA_A subunit isoforms due to the selective oligomerization mediated by receptor assembly, only a limited number of subunit combinations are in fact expressed on the neuronal plasma membrane (71). The majority of GABA_A subtypes in the brain are composed of α1β2γ2, followed by α2β3γ2 and α3β3γ2 with a likely stoichiometry of 2α:2β:1γ, similar to that found for nAChRs (21, 30, 46, 76). To a lesser extent, δ, ε, and π subunits replace the γ subunit to form benzodiazepine-insensitive receptor subtypes, whereas the θ subunit has been shown to replace the β subunit (71). Conversely, ρ subunits (which are expressed predominantly in the retina) rarely coassemble with other GABA_A subunits. Instead they homo- and hetero-oligomerize with other ρ subunits to form a family of pharmacologically distinct GABA-gated chloride channels that have been termed GABA_C receptors (15).

In addition to molecular determination of GABA_A assembly, subunit composition is further restricted by the spatial and temporal pattern of subunit expression. In situ hybridization and immunohistochemical studies have demonstrated that each one of the subunits has a distinct regional and cellular distribution within the brain. Moreover, different subunits and/or subunit combinations further dictate the subcellular localization of these ligand-gated chloride channels and determine their biophysical and pharmacological properties. Detailed knowledge of the molecular composition and the exact anatomical expression of different GABA_A subtypes are therefore crucial in understanding the physiological actions of GABA within the brain and for developing potentially clinically useful subtype-selective drugs that are devoid of the side effects associated with the classical benzodiazepines.

In the cerebellum, the γ2 subunit has been shown to be a component of all postsynaptic GABA_A Rs, whereas the δ subunit is found almost exclusively at extrasynaptic sites (58). GABA_A Rs incorporating a γ2 subunit together with α1–3 subunits (α1–3β2/3γ2) are thus the predominant receptor subtypes responsible for mediating phasic inhibition. It is important to note, however, that these receptor subtypes are also abundant at perisynaptic and extrasynaptic sites. This is consistent with recent evidence for the dynamic mobility and rapid exchange of γ2 subunit-containing receptors between extrasynaptic and synaptic receptor pools (36, 74). Interestingly, α5-containing GABA_A subtypes are found at both extrasynaptic and synaptic sites where they contribute to both phasic and tonic inhibition (1, 19, 20, 66). The α4 and α6 subunits form assembled channel complexes with δ subunits (α4βδ and α6βδ) that are exclusively extrasynaptic, accounting for tonic conductance in the thalamus and cerebellum, respectively (58). On the other hand, the majority of phasic signaling in the cerebellum is due largely to synaptic α6 subunit-containing receptors of the α6β2γ2 combination (58).

IV. FUNCTIONAL SIGNIFICANCE OF GABA_A RECEPTOR STRUCTURAL HETEROGENEITY

While the precise subcellular localization of different GABA_A subtypes undoubtedly contributes to their participation in phasic and tonic forms of signaling, this distinction alone is not sufficient to account for their differential activation. Subunit composition is a major determinant of the binding and gating properties of the ion channels, and hence the magnitude of the response following exposure to ligand. Studies using recombinant GABA_A Rs have revealed
that sensitivity to GABA is defined by the type of α subunit present. Extrasynaptic α6β3δ and α4β3δ subunit compositions display the highest affinities for GABA and synaptic α3β3γ2 subtypes the lowest (14). For both αβγ and αβδ assemblies, the identity of the α subunit also affects the rates of activation, deactivation, and desensitization (11, 20, 32, 49, 53, 75). Conversely, in αβ3γ2 subunit-containing receptors, replacing the γ2 subunit with a δ subunit results in a dramatic reduction in single-channel conductance independent of the type of α subunit present (31). This supports the notion that GABA has a high affinity but low efficacy at δ subunit-containing extrasynaptic receptors. The presence of a γ2 or δ subunit also influences channel kinetics: αβδ receptors desensitize more slowly and less extensively than αβγ receptors (12, 33, 64). Together the distinct biophysical properties of γ and δ subunit-containing receptors are wholly consistent with the involvement of these receptor subtypes in phasic and tonic signaling, respectively.

Differences in subunit composition between synaptic and extrasynaptic receptors are reflected in a differential modulation of phasic and tonic signaling by a number of compounds of therapeutic importance. The most frequently cited example of this is the role of the α subunit in defining receptor affinity for benzodiazepines. GABA<sub>A</sub>Rs incorporating either an α4 or α6 subunit renders receptors insensitive to functional modulation by benzodiazepines (9), as does elimination or substitution of the γ2 subunit. This difference can be attributed solely to the presence of a conserved arginine residue in α4 and α6 subunits, which in α1–3 and α5 subunits is a histidine (88). Thus benzodiazepine site ligands selective for α1–3 subunits largely influence phasic signaling, whereas those selective for α5 subunits are capable of primarily modulating tonic conductance.

The diverse CNS depressant effects of benzodiazepines have been attributed to specific α subunit types of GABA<sub>A</sub>Rs (61). A combined molecular genetic and pharmacological approach has revealed that an α1 subunit-containing receptors mediate the sedative, anamnesic, and, in part, anticonvulsant actions of diazepam, whereas α2 subunits contribute to its anxiolytic and muscle-relaxant effects. Strong pharmacological evidence for the involvement of the α3 subunit in anxiety comes from a study by Atack et al. (3a), who showed that the α3 subtype-selective inverse agonist (α3IA) induces an anxiogenic response in rats. The α5 subunit has been implicated in learning and memory following the observation that a single point mutation (H105R), which prevents the interaction of this receptor subtype with diazepam, abolishes its memory-impairing effects (25). The development of tolerance to the sedative actions of chronic diazepam is also associated with a downregulation of α5 subunit-containing GABA<sub>A</sub>Rs to which α5-H105R mice are resistant (82).

The diverse functions of GABA in the CNS are matched not just by the heterogeneity of GABA<sub>A</sub>Rs but also by the complex trafficking and clustering mechanisms that generate and maintain surface receptor populations accessible to the neurotransmitter at inhibitory postsynaptic specializations or extrasynaptic sites, depending on their subunit composition. The regulation of these mechanisms by GABA<sub>A</sub>R-associated proteins is discussed below.

V. MEMBRANE TRAFFICKING OF GABA<sub>A</sub> RECEPTORS

GABA<sub>A</sub>Rs are not static entities on the neuronal cell surface but are believed to cycle continuously between the plasma membrane and intracellular compartments. The relative rates of receptor exo- and endocytosis are therefore key determinants in controlling the size of the postsynaptic pool accessible to GABA and GABAergic compounds and thus the strength of synaptic inhibition. Importantly, GABA<sub>A</sub>Rs have recently been reported to be inserted into and removed from the plasma membrane exclusively at extrasynaptic sites (13, 74), highlighting the importance of lateral diffusion for their postsynaptic specialization.

VI. EXOCYTOSIS OF GABA<sub>A</sub> RECEPTORS TO THE PLASMA MEMBRANE

GABA<sub>A</sub>Rs can be delivered to the cell surface either as newly assembled channel complexes via a de novo secretory pathway or reinserted following internalization. The oligomerization of GABA<sub>A</sub>R subunits into channel complexes is believed to occur in the endoplasmic reticulum (ER). Evidence suggests that this assembly process plays a critical role in determining the diversity of receptor subtypes expressed on the neuronal plasma membrane. Proteins cannot exit the ER until they have achieved their correctly folded conformation, and misfolded or unassembled proteins are retrotranslocated from this organelle for degradation in the proteasome, restricting the number of subunit combinations that can access the cell surface (42). Following correct assembly, GABA<sub>A</sub>Rs are trafficked to the Golgi apparatus and segregated into vesicles for transport to and insertion into the plasma membrane facilitated by a number of receptor-associated proteins (FIGURE 2).

Yeast two-hybrid screens using the γ2 subunit ICD as bait isolated the first known GABA<sub>A</sub>R associated protein (GABARAP; Ref. 84), a 17-kDa cytosolic protein belonging to the family of membrane-associated proteins (MAPs) involved in membrane trafficking. This includes the estrogen-induced protein first isolated in guinea pig endometrial cells (GEC)-1 (or GABARAP like-1), Golgi-associated transport enhancer of 16 kDa (GATE-16, or GABARAP like-2), Apg8L, and light chain (LC)-3 subunits of MAP1. Interestingly, GABARAP knockout mice do not show differences in
punctate staining of γ2 subunit-containing GABA$\alpha$Rs and lack an overt behavioral phenotype (59). GABARAP may thus be functionally redundant. Nevertheless, the notion that GABARAP is involved in the trafficking of GABA$\alpha$Rs to the plasma membrane (FIGURE 2) is supported by the finding that overexpression of GABARAP in heterologous expression systems and in cultured hippocampal neurons results in an increase in the cell surface expression of γ2 subunit-containing GABA$\alpha$Rs (50). In *Xenopus* oocytes expressing α1β2γ2 subunit-containing GABA$\alpha$Rs, this was accompanied by an increase in GABA$\alpha$R-mediated synaptic inhibition, an effect requiring the γ2 subunit- and microtubule-binding motifs as well as intact polymerized microtubules (23). More recently, it has been demonstrated that GABARAP-mediated exocytosis of GABA$\alpha$Rs is necessary for potentiation of inhibitory transmission by NMDA receptor activation, suggesting that GABARAP may have a role in the regulated delivery of GABA$\alpha$Rs to the plasma membrane after activity rather than in the maintenance of basal surface receptor levels (52).

Evidence in support of a function of GABARAP as a trafficking factor includes the identification of phospholipase C (PLC)-related catalytically inactive protein (PRIP)-1, a 130-kDa protein that is believed to competitively inhibit the binding of the γ2 subunit of GABA$\alpha$Rs to GABARAP (39). PRIP1 knockout mice show impairments in GABA$\alpha$R modulation by benzodiazepines and zinc sensitivity, indicating
reduced activation of γ2 subunit-containing receptors (39). These findings suggest that PRIP1 may play a role in the regulation of GABA<sub>A</sub>R trafficking by GABARAP, ensuring that only mature αβγ receptor complexes are delivered to the plasma membrane (FIGURE 2). In addition, PRIP1 has been shown to directly bind to the ICD of GABA<sub>A</sub>R β1–3 subunits, serving as an adaptor protein for the protein phosphatase (PP)-1α, and as such has been implicated in the phospho-dependent modulation of GABA<sub>A</sub>R functional expression (73). Recently, a second PRIP isoform (PRIP2) has been identified that, like PRIP1, binds both GABARAP and PP1α, suggesting a central role for all PRIP isoforms in modulating GABA<sub>A</sub>R functional expression (80).

GABA<sub>A</sub>Rs also interact with the protein that links the integrin-associated protein with the cytoskeleton (Plic)-1. Plic1 is a 67-kDa protein with a ubiquitin-like (UBL) NH<sub>2</sub>-terminal domain and a ubiquitin-associated (UBA) COOH-terminal domain (45). It is able to bind ubiquitin ligases and components of the proteasome. As such, it is thought to interfere with ubiquitin-dependent proteolysis of proteins (45). Yeast two-hybrid screens and glutathione S-transferase (GST) affinity purification assays have shown that Plic1 interacts with the ICD of α1–3,6 and β1–3 (but not γ2 or δ) subunits of GABA<sub>A</sub>Rs (7), indicating that Plic-1 function may be relevant for the majority of receptor subtypes expressed in the brain. This interaction has been demonstrated to be of significance in mediating the functional expression of GABA<sub>A</sub>Rs in human embryonic kidney (HEK-293) cells and in hippocampal slices as revealed using dominant negative peptides (7). In a recent study by Saliba et al. (63), the authors showed that Plic1 increases the accumulation of GABA<sub>A</sub>R β3 subunits on the cell surface in a manner independent of their rates of internalization (FIGURE 2). These findings suggest that Plic1 selectively modulates the secretory pathway. In accordance with this, Plic1 was found to significantly increase the half-life of polyubiquitinated GABA<sub>A</sub>R β3 subunits in the ER and to facilitate their insertion into the plasma membrane (63). By increasing the residence times of unassembled subunits in the ER, Plic1 may also increase subunit maturation and production of heteromeric receptors. Plic1 regulation of the ubiquitin-dependent proteasomal degradation of GABA<sub>A</sub>Rs may thus provide a dynamic mechanism for regulating the efficacy of inhibitory synaptic transmission.

The 190-kDa brefeldin A-inhibited guanine nucleotide exchange factor (BIG)-2 was also identified as a GABA<sub>A</sub>R β subunit interacting protein in a yeast two-hybrid screen (22). BIG2 is concentrated mainly in the trans-Golgi network but is also found in vesicle-like structures along dendrites and at the postsynaptic plasma membrane (FIGURE 2; Ref. 22). Interestingly, coexpression of BIG2 with the GABA<sub>A</sub>R β3 subunit results in an increase in β3 exit from the ER, suggesting that BIG2 is involved in the post-Golgi vesicular trafficking of GABA<sub>A</sub>Rs (22). It is currently proposed that BIG2 may play a role in the transport of newly assembled GABA<sub>A</sub>Rs to the postsynaptic plasma membrane and also be involved in receptor recycling (67, 68). Other GABA<sub>A</sub>R-associated proteins implicated in the forward trafficking of GABA<sub>A</sub>Rs include the GABA<sub>A</sub>R interacting factor (GRIF)-1 (6) and the multifunctional protein gClqQR (65). However, their functional significance remains unclear.

VII. ENDOCYTOSIS OF GABA<sub>A</sub> RECEPTORS FROM THE PLASMA MEMBRANE

GABA<sub>A</sub>Rs have been shown to be localized in clathrin-coated pits, suggesting that they undergo clathrin-mediated endocytosis, a process that is further dependent on dynamin for endocytic vesicle formation. The clathrin adaptor protein (AP)-2 is a central component in the formation of these vesicles, forging a link between membrane proteins and clathrin, which forms the outer layer of the coat. AP2 is a heterotetrameric complex composed of two large (∼100 kDa) α and β2 subunits, a medium (50 kDa) μ2 subunit, and a small (19 kDa) σ2 subunit, commonly referred to as adaptins.

GABA<sub>A</sub>Rs are intimately associated with AP2 in the brain through a direct binding of the β1–3 and γ2 GABA<sub>A</sub>R subunits (41). In the GABA<sub>A</sub>R β2 subunit, a dileucine AP2-β2 adaptin-binding motif (L<sup>144</sup>L<sup>145</sup>) has been identified. This motif is important for clathrin-mediated endocytosis in HEK-293 cells and in cortical slices (32, 33). It is also present in the ICDs of receptor β1 and β3 subunits, but evidence suggests that it is not involved in the interaction of these subunits with the AP2 complex (39). In addition, an atypical AP2-binding motif conserved within the ICD of all GABA<sub>A</sub>R β subunit isoforms has been identified (KTHLR RRRSQLK in the β3 subunit) (39). This motif, which is enriched in lysine and arginine residues, also incorporates the major sites of phosphorylation for cAMP-dependent protein kinase (PKA) and calcium/phospholipid-dependent protein kinase (PKC) within this class of receptor subunits: S<sup>409</sup> in β1, S<sup>410</sup> in β2, and S<sup>408/9</sup> in β3 (56). In vitro binding experiments have shown that phosphorylation and/or mutation of these residues confers a reduction in binding of the GABA<sub>A</sub>R β subunits to the μ2 adaptin of the AP2 complex (FIGURE 2; Refs. 35, 39). Moreover, neurons expressing GABA<sub>A</sub>Rs incorporating fluorescent β3<sup>S408/9A</sup> subunits exhibit reduced endocytosis and enhanced functional expression at both synaptic and extrasynaptic sites (35). Intriguingly, while analyzing the cell surface distributions of wild-type and S408/9A mutant β3 subunit-containing GABA<sub>A</sub>Rs, the authors noted an apparent increase in long, filopodia-like spines on neurons expressing the latter. This deficit in spine maturity was reversed by pharmacological blockade of GABA<sub>A</sub>Rs. Regulating the efficacy of synaptic inhibition by modulating GABA<sub>A</sub>R membrane trafficking may thus play a critical role in regulating spinogenesis and synaptic plasticity (35).
More recently, a tyrosine-based AP2-μ2 adaptin-binding motif in the GABA<sub>A</sub>R γ2 subunit (Y<sup>365</sup>GYY<sup>367</sup>C) has been identified that is also conserved in the γ1 and γ3 subunits (40). These tyrosine residues are the principal sites for phosphorylation by Fyn and Src kinase (13, 36, 57). Utilizing nonphosphorylated and phosphorylated peptides corresponding to Y365 and Y367, the authors showed that this high-affinity interaction is phospho-dependent (40). Introduction of the nonphosphorylated γ2 peptide into neurons induced a large increase in the miniature inhibitory postsynaptic current (mIPSC) amplitude that was accompanied by an increase in the number of receptors on the cell surface (40).

The physiological significance of GABA<sub>A</sub>R endocytosis has been further evaluated by creating a knock-in mouse in which Y365 and Y367 in the γ2 subunit have been mutated to phenylalanine, significantly increasing the affinity for AP2-μ2. Homozygotes for these mutations die in utero. Heterozygotes are viable and do not exhibit any gross behavioral deficits. Consistent with the roles of Y365/7F in mediating high-affinity binding to AP2-μ2 Y365/7F, knock-in mice have elevated total and cell surface expression levels. In the hippocampus this results in an increase in the size of inhibitory synapses and efficacy of synaptic inhibition that is specific to CA3. This increase in inhibition correlates with a specific deficit in spatial memory in Y365/7F mice, a behavioral paradigm dependent on CA3. Modifying GABA<sub>A</sub>R endocytosis clearly has behavioral consequences.

VIII. POSTENDOCYTIC GABA<sub>A</sub> RECEPTOR SORTING

The fate of internalized receptors is another determinant of surface receptor levels. Following internalization, GABA<sub>A</sub>Rs are either rapidly recycled back to the neuronal plasma membrane or, over longer time frames, are targeted for lysosomal degradation, an endocytic sorting decision that is regulated by the Huntingtin-associated protein (HAP)-1. Yeast two-hybrid screens have revealed that HAP1 interacts with the GABA<sub>A</sub>R β1 subunit (Figure 3) but not the α1, γ2, or δ subunits (44). Overexpression of HAP1 in cultured neurons has been shown to increase the number of receptors on the cell surface at steady state, an increase that correlates with a dramatic increase in the mean amplitude of mIPSCs but that does not affect the frequency or kinetics of these events (44). However, whether HAP1 promotes GABA<sub>A</sub>R recycling or prevents receptor lysosomal degradation is an unresolved issue. Nevertheless, the importance of HAP1-dependent regulation of GABA<sub>A</sub>R trafficking is clear, as evidenced by a study in which selective suppression of hypothalamic HAP1 by siRNA induced a decrease in feeding behavior in mice that was attributed to reduced surface expression and activity of GABA<sub>A</sub>Rs (69). More recently it was established that HAP1 plays a role in regulating the targeting of GABA<sub>A</sub>Rs to inhibitory synapses dependent on the molecular motor-KIF5 (80).

IX. POSTSYNAPTIC TARGETING AND CLUSTERING OF GABA<sub>A</sub> RECEPTORS

The highly selective subcellular localization of GABA<sub>A</sub>R subtypes implies that subunit composition plays a major role in the postsynaptic targeting and clustering of these receptors. While the exact molecular mechanisms that govern the accumulation of GABA<sub>A</sub>Rs at inhibitory synapses are not yet fully understood, it involves a number of receptor-associated proteins and cytoskeletal elements that are concentrated at postsynaptic densities (PSDs).

The inhibitory synaptic marker gephyrin is a 93-kDa sub-synaptic scaffolding protein that was originally implicated in regulating the postsynaptic clustering of glycine receptors in the spinal cord by directly binding to the receptor β subunit (35). More recently, data derived from gephyrin knockout mice and knockdown experiments using antisense oligonucleotides or shRNAi have revealed that reducing gephyrin expression also leads to an extensive loss in the punctate staining of GABA<sub>A</sub>Rs incorporating a γ2 subunit together with α2 (but not α1) subunits (23, 34, 46, 51). This suggests the existence of both gephyrin-dependent and -independent GABA<sub>A</sub>R clustering mechanisms. Gephyrin clusters are absent in γ2 subunit knockout mice. The remaining α and β subunit-containing receptors show diffuse staining (28). Furthermore, transfection of γ2 subunit-deficient neurons with chimeric α2/γ2 constructs revealed that the TM4 of the γ2 subunit is sufficient for targeting these receptors to postsynaptic sites, but that both the TM4 and ICD of the γ2 subunit were necessary for recruiting gephyrin to the synapse and rescuing GABAergic inhibitory synaptic function (2). A role for this receptor-associated protein in stabilizing previously clustered GABA<sub>A</sub>Rs at the cell surface, rather than their specialization at inhibitory synapses, has thus been proposed. Concurrent with this, postsynaptic GABA<sub>A</sub>Rs have been shown to be three times more mobile in cells where gephyrin expression has been impaired as measured by fluorescence recovery after photobleaching (34). It is emerging that the rate of lateral mobility of GABA<sub>A</sub>Rs plays a central role in determining the number of these receptors at synaptic sites, a process revealed by the use of single particle tracking. This approach has illustrated that there is rapid exchange between extrasynaptic and synaptic populations of GABA<sub>A</sub>Rs and that this process is subject to modulation via the activity of protein phosphatase 2B (PP2B). This calcium-dependent mechanism activated via NMDA receptors leads to an increase in the lateral mobility of GABA<sub>A</sub>Rs and a reduction in the size of inhibitory synapses, a process that favors neuronal depolarization (4).

Gephyrin is believed to anchor postsynaptic GABA<sub>A</sub>Rs to the plasma membrane by cross-linking the receptor mole-
Gephyrin binds with high affinity to tubulin and as such is viewed as a bona fide microtubule-binding protein MAP (61). Gephyrin also mediates an indirect interaction with the cytoskeleton by binding to LC1 and LC2 of the dynein motor proteins and the microfilament-associated proteins belonging to the Mena/VASP family (27). The membrane-associated protein collybistin II is a guanine-nucleotide exchange factor (GEF) that binds to and so permits the subsynaptic localization of the otherwise intracellular gephyrin, where it directly interacts with and immobilizes specific GABA$_A$R subtypes on the plasma membrane (30, 38). In addition, the dystrophin glycoprotein complex (DGC) and neuroligin (NL)-2 bridge the synaptic cleft by interacting with presynaptic $\beta$-neurexin, promoting GABAergic synaptogenesis (78).

Although gephyrin is found to be concentrated at GABAergic synapses in both hippocampal and cortical neurons (where...
glycine receptor expression is relatively low), in initial studies using standard biochemical methods it failed to copurify with any of the GABA\(_A\)R subunits. Hence, an intermediate linker protein was postulated. Given its ability to directly bind the \(\gamma_2\) subunit ICD and gephyrin, the prime candidate was GABARAP. However, subsequent analysis revealed that GABARAP is not clustered at gephyrin-rich postsynaptic specializations but instead is mainly localized in intracellular compartments including the ER, Golgi apparatus and, to some extent, subsynaptic secretory vesicles (43, 84). This is consistent with a role for this protein in intracellular trafficking rather than postsynaptic clustering.

In a recent study Tretter et al. (77) for the first time provided evidence that gephyrin binds directly to the \(\alpha_2\) subunit ICD in vitro. This interaction was blocked by low concentrations of detergent, providing a possible explanation as to why previous attempts to identify a direct association between gephyrin and GABA\(_A\)Rs were unsuccessful. However, under the same conditions, only very weak binding to \(\beta_3\) and \(\gamma_2\) subunits was observed (77), suggesting that \(\alpha\) subunit isoforms may play a predominant role in the synaptic accumulation of GABA\(_A\)Rs via their ability to directly bind gephyrin.

The 81-kDa actin-binding protein radixin, a member of the ezrin/radixin/moesin protein family, has recently been shown to directly link the \(\alpha_5\) subunit to the cytoskeleton via a radixin-binding motif conserved within the ICD of \(\alpha_1–3\) and \(\alpha_5\) subunits, differing only by two amino acids in the \(\alpha_2\) subunit (52). In neurons, both depletion of radixin and replacement of the radixin actin-binding motif dramatically decreased \(\alpha_5\) subunit-containing GABA\(_A\)R clusters without altering total surface expression levels. Radixin also showed limited colocalization with postsynaptic gephyrin, consistent with previous reports that GABA\(_A\)R \(\alpha_5\) subunits localize mainly at extrasynaptic sites (14, 52). However, the mechanisms responsible for the formation of radixin-dependent extrasynaptic GABA\(_A\)R clusters remain to be elucidated.

### X. MODULATION OF GABA\(_A\) RECEPTOR FUNCTION BY POSTTRANSLATIONAL MODIFICATIONS

The cell surface stability of GABA\(_A\)Rs is further regulated by posttranslational modifications such as palmitoylation, ubiquitination, and phosphorylation. These modifications have been implicated in altering the biophysical and pharmacological properties of these ligand-gated ion channels (3).

#### A. Palmitoylation

Palmitoylation is the covalent attachment of the saturated fatty acid palmitate to cysteine residues of a given protein by the palmitoyltransferase Golgi-specific DHHC zinc finger domain protein (GODZ). Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. As such it has been shown to be involved in the postsynaptic clustering and subcellular trafficking of a number of proteins, including AMPA receptors (31) and the neuronal scaffold proteins PSD-95 and glutamate receptor-interacting protein (GRIP)-1 (20, 74). It is unique in that it is the only reversible lipid modification and thus allows the cell to dynamically regulate the location of specific proteins. In a yeast two-hybrid screen, the 34-kDa protein GODZ was identified as a GABA\(_A\)R \(\gamma_2\) subunit-interacting protein that recognizes a 14-amino acid cysteine-rich domain conserved in the ICD of all \(\gamma_1–3\) subunits, NH\(_2\)-terminal to the GABARAP binding site (60). Analysis of Cys-Ala mutant \(\gamma_2\) constructs in transfected COS-7 cells revealed that the \(\gamma_2\) subunit is palmitoylated at all four cysteines within the GODZ binding domain (60). Mutation of these cysteine residues resulted in a loss of GABA\(_A\)R clusters at the cell surface, as did drug-induced global inhibition of palmitoylation by Br-palmitate (60). Likewise, disrupting GODZ function or expression levels using dominant negative or RNAi approaches resulted in a significant reduction in the amplitude of mIPSCs attributed to a decrease in postsynaptic GABA\(_A\)R number (24). From these studies it is evident that palmitoylation can dynamically regulate the efficacy of neuronal inhibition by controlling the accumulation of GABA\(_A\)Rs at the postsynaptic membrane, although the exact mechanism by which this is achieved is unknown.

#### B. Ubiquitination

The regulation of GABA\(_A\)R trafficking by the ubiquitin-related protein Plc1 suggests that GABA\(_A\)Rs may also be direct targets for modification by the polypeptide ubiquitin. The covalent attachment of one or more copies of the 76-amino acid ubiquitin monomer to lysine residues of target proteins is referred to as ubiquitination. Monoubiquitination is reversible and serves as an active signal in diverse intracellular trafficking pathways, including as a trigger for endocytosis. In contrast, polyubiquitination is required for the translocation of proteins from the ER back into the cytosol, where they are degraded by the proteasome. Activity-dependent polyubiquitination of GABA\(_A\)R \(\beta_3\) subunits has been shown to reduce the stability of newly translated and assembled receptors in the ER via a mechanism dependent on the activity of the proteasome (62).

Coincident with a loss of cell surface expression levels, chronic blockade of neuronal activity by tetrodotoxin (TTX) treatment reduced both the amplitude and frequency of mIPSCs (62). TTX had no effect on the enhanced functional expression of GABA\(_A\)Rs incorporating \(\beta_3\) subunits in which all 12 lysine residues within the ICD of this subunit had been mutated to arginines (\(\beta_3^{K12R}\)). These mutations
did not alter GABA<sub>δ</sub>R cell surface half-life or internalization rates but did significantly enhance receptor insertion into the plasma membrane (62).

C. Phosphorylation

Protein phosphorylation is fundamental to the activity of cellular signaling networks. It is achieved through protein kinase-catalyzed transfer of a phosphate group to serine, threonine, and/or tyrosine residues of a given protein substrate that can be reversed in a dephosphorylation reaction catalyzed by protein phosphatases. GABA<sub>δ</sub>Rs are well-established phosphoproteins. Diverse studies on GABA<sub>δ</sub>R phosphorylation have implicated this process in altering channel gating, conductance and/or kinetics, sensitivity of the receptors to pharmacological agents, protein-protein interactions, and membrane trafficking (83). Hence, the coordinated activity of kinases and phosphatases plays a pivotal role in controlling neuronal excitability.

Studies in heterologous expression systems have demonstrated that GABA<sub>δ</sub>R function, depending on the subtype analyzed, can be differentially modulated by phosphorylation of key residues within the ICDs of receptor β1–3 and γ2 subunits by a number of kinases, including PKA, PKC, calcium/calmodulin-dependent kinase II (CaMKII), protein kinase B (PKB; also known as Akt), cGMP-dependent protein kinase (PKG), and tyrosine kinases of the Src family (summarized in Table 1). This is best illustrated by the differential modulation of GABA<sub>δ</sub>R subtypes by PKA, depending on the identity of the β subunit. In vitro studies using purified bacterially expressed GST fusion proteins combined with site-directed mutagenesis revealed that the consensus motif for PKA-induced phosphorylation conserved within the ICDs of GABA<sub>δ</sub>R β1–3 subunits is RRRXSQLK, where S is a serine at position 409 in β1 and β3 subunits and at position 410 in the β2 subunit (55) and X represents either an alanine residue in β1 and β2 subunits or a serine residue at position 408 in the β3 subunit. However, in contrast to in vitro findings, analysis of recombinant GABA<sub>δ</sub>Rs in HEK-293 cells has demonstrated that PKA-induced phosphorylation of the β3 subunit occurs at both S408 and S409 (rather than at S409 alone), whereas the β2 subunit is not a substrate for this kinase in heterologous expression systems (54). Interestingly, PKA was shown to depress GABA-activated currents in HEK-293 cells expressing β1 subunit-containing GABA<sub>δ</sub>Rs, whereas in β3 subunit-expressing cells, a potentiation was observed (54). The latter was attributed to the presence of two juxtaposed serine residues (S408 and S409) in β3, as selective mutation of S408 to alanine (to structurally resemble the GABA<sub>δ</sub>R β1 subunit at this site) converted this potentiation to a depression (54). On the other hand, PKA had no effect on β2 subunit-expressing cells (54), which can be accounted for by the selective recruitment of PKA to GABA<sub>δ</sub>R β1 and β3, but not β2, subunits via the A-kinase anchoring protein (AKAP) of 79 (rat)/150 (human) kDa (18).

AKAPs have also been shown to interact directly with PP2B and PKC in addition to PKA (24, 44); however, the targeting of PP2B and PKC to GABA<sub>δ</sub>Rs by AKAP remains to be investigated. Interestingly, PP2B has been shown to bind directly to the ICD of the γ2 subunit, thereby dephospho-

<table>
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<th>Subunit</th>
<th>Phosphorylation Site</th>
<th>Protein Kinase</th>
<th>In vitro</th>
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<th>Primary neurons</th>
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<tr>
<td>β1</td>
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PKA, cAMP-dependent protein kinase; PKC, calcium/phospholipid-dependent protein kinase; CaMKII, calcium/calmodulin-dependent kinase II; PKB, protein kinase B (also known as Akt); PKG, cGMP-dependent protein kinase; Src, and tyrosine kinases of the Src family. [Adapted from Brandon et al. (18).]
rminating the receptor and inducing long-term depression (LTD) of synaptic inhibition in the CA1 region of the hippocampus, a mechanism that is believed to contribute to learning and memory (85). PKC is recruited to the GABA\textsubscript{A}R via the anchoring protein receptor for activated C kinase (RACK)-1. Like PKA, it has different effects on receptor function depending on subunit composition. In agreement with the findings in vitro, all of the β subunit isoforms were found to be a substrate for this kinase in HEK-293 cells (17, 48, 54). Functional studies employing site-directed mutagenesis have demonstrated a downregulation of receptor activity in response to phorbol esters mediated by PKC-induced phosphorylation of β1 S409, β2 S410, γ2S S327, and γ2L S327/343. However, the use of a constitutively active catalytic domain of PKC (PKM) produced results contradicting those obtained with phorbol esters. In transfected mouse fibroblasts, PKM enhanced the response to GABA in a manner that was dependent on the phosphorylation of the β1 subunit at S409 and the γ2L subunit at S327 and S343 (51). The reason for this discrepancy remains unresolved, but it has been speculated that different PKC isoforms may produce different responses (72). In contrast, brain-derived neurotrophic factor (BDNF)-induced PKC-mediated phosphorylation of the β3 subunit has been shown to transiently increase the amplitude of mIPSCs in cultured neurons, an effect paralleled by an increase in whole cell GABA-activated currents in HEK-293 cells, an effect abolished by site-specific mutagenesis of both of these tyrosine residues to phenylalanines (56). Interestingly, mutation of these sites led to increased phosphorylation of the β1 subunit at Y384 and Y386, a subunit that exhibits relatively low stoichiometry of phosphorylation in response to Src compared with wild-type control (56). In primary neuronal cultures, intracellular application of sodium vanadate, a potent tyrosine phosphatase inhibitor, enhanced benzodiazepine-sensitive GABA\textsubscript{A}R function, suggesting high endogenous tyrosine kinase and phosphatase activity under basal conditions (56). Consistent with this, studies using phospho-antibodies have revealed that γ365/7 are principally phosphorylated via the activity of Fyn and Src. Significantly, Fyn is able to bind specifically to phospho-Y367, providing a mechanism for the recruitment of this tyrosine kinase to GABA\textsubscript{A}Rs (16, 38). Studies with phospho-antibodies have revealed significant variations in the stoichiometry of Y367 phosphorylation in the brains of rodents, suggesting input-specific control of phosphorylation; however, the signaling pathways that regulate the phosphorylation of the γ2 subunit remain to be defined. In keeping with their roles in mediating high-affinity binding to AP2, phosphorylation of Y365/7 increases the cell surface stability of GABA\textsubscript{A}Rs and enhances the amplitude and frequency of mIPSCs (40, 79).

### XI. CONCLUDING REMARKS

GABA\textsubscript{A}Rs play a central role in mediating neuronal inhibition and mediating the effects of a broad range of anticonvulsant, anxiolytic, and sedative-hypnotic agents. GABA\textsubscript{A}Rs mediate both phasic and tonic modes of inhibition, phenomena that are mediated via receptor subtypes with distinct molecular structures. Phasic inhibition is mediated largely by benzodiazepine-sensitive receptor subtypes that are highly enriched at synaptic sites and are principally assembled from α1–3, β1–3, and γ2 subunits. A critical determinant of receptor number at inhibitory synapses is the steady-state cell levels of these receptors on the neuronal plasma membrane. This is likely determined by the relative rates of receptor exo- and endocytosis, processes that are intimately controlled by covalent modifications and interaction with specific binding partners. While the molecular details of these processes are being evaluated, their significance for the ef-
ficiency of neuronal inhibition and ultimately for behavior remains to be established. Our comprehension of how GABA\textsubscript{A}Rs are selectively stabilized at inhibitory synapses is limited; however, a central role for gephyrin is emerging. The direct binding of gephyrin to the GABA\textsubscript{A}R subtypes containing \(\alpha 2\) subunits has recently been shown (77). The significance of this interaction for GABA\textsubscript{A}R subtypes containing other \(\alpha\)-subunit isoforms warrants further investigation. Gephyrin-independent clustering mechanisms have also been postulated, in particular for subtypes containing \(\alpha 5\) subunits. The importance of these emerging processes for other receptor subtypes remains to be demonstrated.

The cellular mechanisms that regulate the cell surface accumulation are under active investigation. Future studies to ascertain the significance of these molecular mechanisms in determining the efficacy of neuronal inhibition under both normal and pathological conditions are essential.

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**DISCLOSURES**

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