$GABA_A$ receptor trafficking and its role in the dynamic modulation of neuronal inhibition

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Abstract | GABA (γ-aminobutyric acid) type A receptors ($GABA_A$Rs) mediate most fast synaptic inhibition in the mammalian brain, controlling activity at both the network and the cellular levels. The diverse functions of GABA in the CNS are matched not just by the heterogeneity of $GABA_A$Rs, but also by the complex trafficking mechanisms and protein–protein interactions that generate and maintain an appropriate receptor cell-surface localization. In this Review, we discuss recent progress in our understanding of the dynamic regulation of $GABA_A$R composition, trafficking to and from the neuronal surface, and lateral movement of receptors between synaptic and extrasynaptic locations. Finally, we highlight a number of neurological disorders, including epilepsy and schizophrenia, in which alterations in $GABA_A$R trafficking occur.

Tonic inhibition
An inhibitory response that results from the activation of extra- or perisynaptic $GABA_A$ receptors by ambient concentrations of GABA.

Benzodiazepines
Pharmacologically active molecules with sedative, anxiolytic, amnesic and anticonvulsant effects. They act by binding at the interface between the α (1, 2, 3 or 5) and γ subunits of $GABA_A$ receptors and potentiating the response elicited by GABA.

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Synaptic inhibition in the brain is largely a result of GABA (γ-aminobutyric acid) signalling. The fast inhibitory actions of GABA are mediated by the activation of $GABA_A$ receptors ($GABA_A$Rs) in the brain[1,2] and $GABA_C$ receptors in the retina[3], whereas its slow, prolonged actions are mediated by metabotropic G-protein-coupled $GABA_B$ receptors[4,5]. $GABA_A$Rs are also clinically relevant drug targets for anti-convulsant, anxiolytic and sedative–hypnotic agents. Moreover, deficits in the functional expression of $GABA_A$Rs are critical in epilepsy, anxiety disorders, cognitive deficits, schizophrenia, depression and substance abuse. Understandably, there has been considerable interest in determining the cellular mechanisms that regulate $GABA_A$R accumulation on the neuronal plasma membrane.

Molecular studies have demonstrated that $GABA_A$Rs are part of a ligand-gated ion-channel superfamily, other members of which include nicotinic acetylcholine receptors, glycine receptors and 5-hydroxytryptamine 3 receptors[6,7]. Proteins belonging to this superfamily are heteropentamers that are assembled from a range of homologous subunits that share a common structure: a large amino-terminal extracellular domain and four transmembrane domains (TMs), with a large intracellular domain between TM3 and TM4 (FIG. 1a). To date, 18 $GABA_A$R subunits have been identified. Based on sequence homology, these are divided into seven subunit classes, some of which have multiple members: α (1–6), β (1–3), γ (1–3), δ, ε (1–3), θ and π. $GABA_A$R structural diversity is further increased by the alternative splicing of some receptor mRNAs. However, most $GABA_A$Rs are composed of two α subunits, two β subunits and one γ (or one δ) subunit[2] (FIG. 1b).

$GABA_A$Rs with different subunit composition have different physiological and pharmacological properties, are differentially expressed throughout the brain and are targeted to different subcellular regions. For instance, receptors composed of α1, α2, α3 or α5 subunits together with β and γ subunits are benzodiazepine-sensitive, are largely synaptically located and mediate most phasic inhibition in the brain[2] (with the notable exception of extrasynaptically localized α5-containing receptors[8,9]) (FIG. 1c). By contrast, those composed of α4 or α6 subunits together with β and δ subunits make up a specialized population of predominantly extrasynaptic receptor subtypes that mediate tonic inhibition and are insensitive to benzodiazepine modulation[9]. In addition, there are also $GABA_A$Rs at presynaptic sites[10].

Here we address how neurons regulate the assembly, membrane trafficking, synaptic accumulation and function of these distinct $GABA_A$R subtypes and the relevance of these emerging regulatory processes for the efficacy of neuronal inhibition in both health and disease.

Ubiquitin–proteasome system
(UPS). Ubiquitin is a 76-amino-acid protein that, among other functions, tags proteins for degradation. Tagged proteins are targeted to the proteasome, a large, multimeric barrel-like complex that degrades proteins.

Controlling $GABA_AR$ assembly

$GABA_ARs$ are assembled from their component subunits in the endoplasmic reticulum (ER). This process has a critical role in determining the diversity of $GABA_ARs$ that are expressed on the neuronal cell surface, because exit from the ER is dependent on proteins reaching 'conformation maturity', and misfolded proteins are retro-translocated from this organelle for degradation in the proteasome.

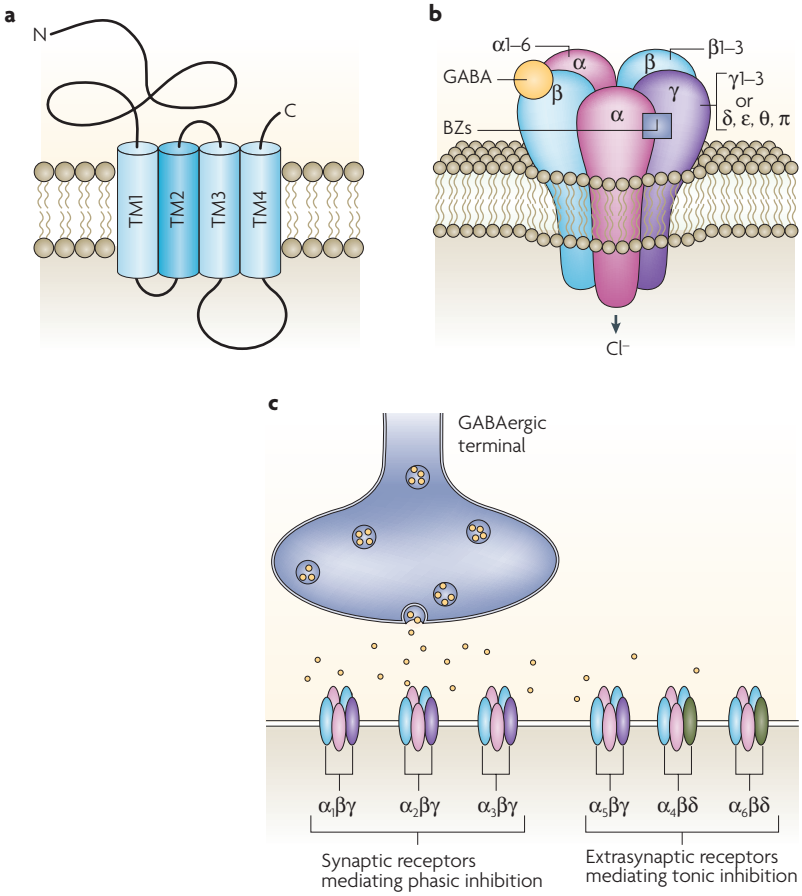

Figure 1 | **$GABA_A$ receptor structure and neuronal localization. a** | GABA (γ-aminobutyric acid) type A receptors ($GABA_ARs$) are members of the ligand-gated ion-channel superfamily. $GABA_AR$ subunits consist of four hydrophobic transmembrane domains (TM1–4), with TM2 believed to line the pore of the channel. The large extracellular amino terminus is the site of GABA binding, and also contains binding sites for psychoactive drugs, such as benzodiazepines (BZs). Each receptor subunit also contains a large intracellular domain between TM3 and TM4 that is the site for various protein interactions as well as for various post-translational modifications that modulate receptor activity. **b** | Five subunits from seven subunit subfamilies (α, β, γ, δ, ε, θ and π) assemble to form a heteropentameric Cl^--permeable channel. Despite the extensive heterogeneity of the $GABA_AR$ subunits, most $GABA_ARs$ expressed in the brain consist of two α subunits, two β subunits and one γ subunit; the γ subunit can be replaced by δ, ε, θ or π. Binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, allowing the rapid influx of Cl^- into the cell. BZ binding occurs at the interface between the α (1, 2, 3 or 5) and γ subunits and potentiates GABA-induced Cl^- flux. **c** | $GABA_ARs$ composed of α (1–3) subunits together with β and γ subunits are thought to be primarily synaptically localized, whereas α5βγ receptors are located largely at extrasynaptic sites. Both these types of $GABA_AR$ are BZ sensitive. By contrast, receptors composed of α(4 or 6)ββδ are BZ insensitive and localized at extrasynaptic sites. Part **a** reproduced, with permission, from REF. 144 © (2001) Macmillan Publishers Ltd. Part **b** reproduced, with permission, from REF. 145 © (2005) Macmillan Publishers Ltd.

Limiting diversity through selective oligomerization. Many different subunit combinations are theoretically possible; however, studies reveal that only a limited number of these combinations can actually exit the ER and access the neuronal cell surface. The majority of studies agree that most $GABA_ARs$ expressed on the surface of neurons are composed of two α subunits, two β subunits and one γ subunit (although the γ subunit can be replaced by a δ, an ε, a θ or a π subunit depending on the neuron type and the subcellular localization of the receptor)[2,11]. Most homomeric subunits, and αγ and βγ heteromers, are retained in the ER and degraded (for a review, see REF. 12). Thus, the expression and assembly of these subunits must be carefully regulated in the ER, by mechanisms that involve classical ER-resident chaperones, such as heavy-chain binding protein and calnexin[13].

Sequences in the N terminus of $GABA_AR$ subunits control receptor oligomerization and thus promote the assembly of particular subunit combinations[12]. The oligomerization of individual $GABA_AR$ subunits into heteromers occurs within 5 minutes of translation[14]. However, this process is inefficient, and less than 25% of translated subunits are assembled into heteromeric receptors[14]. $GABA_AR$-subunit-deficient mice have provided insights into the preferential assembly of select $GABA_ARs$ *in vivo*. For example, loss of the δ subunit from the plasma membrane of cerebellar granule cells is observed in α6-knockout mice[15]. Similarly, there is a decrease in the levels of the α4 subunit in the forebrain of δ-subunit-deficient mice, whereas the levels of the α1 subunit remain unchanged[16,17]. This indicates that the δ subunit preferentially assembles with α4 and α6 subunits. There is also a compensatory increase in γ2 subunit levels in δ-subunit-deficient mice[16,17], suggesting that the γ2 subunit associates with the α4 subunit in the absence of the δ subunit. These findings suggest that subunits compete to find their preferential oligomerization partners in the ER. However, the details of these processes remain to be determined.

Activity-dependent $GABA_AR$ ubiquitylation. The ER is responsible for the retention and degradation of misfolded or unassembled subunits and, accordingly, homomeric unassembled $GABA_AR$ subunits have been shown to be degraded in this organelle[14,18]. ER-associated degradation (ERAD) involves protein ubiquitylation and degradation by the ubiquitin–proteasome system (UPS)[19]. $GABA_AR$ subunits have recently been shown to be ubiquitylated in an activity-dependent manner[20]. Chronic blockade of neuronal activity dramatically increased the levels of $GABA_AR$ ubiquitylation in the ER, resulting in decreased insertion at the plasma membrane[20]. Correspondingly, increasing neuronal activity resulted in a decrease in the level of $GABA_AR$ ubiquitylation and an enhancement of receptor cell-surface expression[20]. Thus, neuronal activity can regulate the ubiquitylation of $GABA_ARs$ in the ER, affecting their rate of degradation by the UPS. This might be one mechanism that neurons use to homeostatically regulate synaptic inhibition.

The fate of ubiquitylated $GABA_A$Rs is also likely to be modulated by their association with the ubiquitin-like proteins PLIC1 and PLIC2 (REF. 18), which have been demonstrated to block the degradation of ubiquitylated substrates[21]. PLIC1 binds to the intracellular domain of α and β $GABA_A$R subunits through its ubiquitin-associated domain[18]. It increases the half-life of $GABA_A$Rs, resulting in an increase in the number of receptors that are available for insertion into the plasma membrane[18]. PLIC1 does not affect the rate of receptor endocytosis[18]; rather, it seems to function solely in the secretory pathway, stabilizing $GABA_A$Rs and/or inhibiting their degradation by the UPS and thereby facilitating their accumulation at inhibitory synapses.

Facilitating $GABA_A$R trafficking

After their assembly in the ER, transport-competent $GABA_A$Rs are trafficked to the Golgi apparatus and segregated into vesicles for transport to, and insertion into, the plasma membrane. Our understanding of these processes remains rudimentary, but it is becoming clear that they are facilitated by a number of receptor-associated proteins (FIG. 2, TABLE 1); these proteins are described in the following sections.

GABARAP and NSF. $GABA_A$ receptor-associated protein (GABARAP) interacts with the intracellular domain of $GABA_A$R γ subunits *in vitro* and *in vivo*[22]. It also binds to microtubules[23] and to *N*-ethylmaleimide-sensitive factor (NSF)[24], a protein that is involved in intracellular vesicular fusion events[25]. GABARAP is concentrated in the Golgi apparatus and in intracellular vesicles, but it is not present at GABAergic synapses[22,24,26], suggesting that its main role is in the intracellular transport of $GABA_A$Rs. Overexpressing GABARAP with $GABA_A$Rs results in increased cell-surface receptor expression, possibly owing to enhanced intracellular receptor trafficking[27–29]. This effect can be abolished by a mutation that disrupts the addition of phospholipids to GABARAP[30], apparently increasing its membrane association. The addition of phospholipids is thus critical for GABARAP to control $GABA_A$R trafficking[30]. Analysis of GABARAP-knockout mice did not reveal any alterations in synaptic γ2-containing $GABA_A$R levels[31]; however, this might reflect redundancy, given the existence of other GABARAP homologues that can interact with $GABA_A$Rs[32]. Recently it was demonstrated that GABARAP is necessary for increasing cell-surface $GABA_A$R expression after NMDA (*N*-methyl-D-aspartate) receptor activation[33], suggesting that GABARAP might have a role in the regulated delivery of $GABA_A$Rs to the surface after activity, rather than in the maintenance of basal receptor levels.

NSF has also been found to bind directly to $GABA_A$R β subunits[34]. NSF and GABARAP might act together to promote the forward trafficking of $GABA_A$Rs from the Golgi apparatus. Indeed, the subcellular distribution of both $GABA_A$Rs and NSF is disturbed when the lipid modification of GABARAP is prevented in neurons, resulting in fewer $GABA_A$Rs being trafficked to the plasma membrane[30]. However, another study found that overexpression of NSF significantly reduced $GABA_A$R cell-surface levels in both heterologous systems and neurons[34]. This effect on $GABA_A$R is opposite to that which is observed when GABARAP is overexpressed[27–29] and is also opposite to NSF's role in enhancing AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor surface expression[35,36]. This might indicate that NSF has additional functions in the endocytic pathway; however, further studies are required to understand exactly how NSF regulates $GABA_A$R levels.

PRIPs. Phospholipase-C-related catalytically inactive proteins (PRIPs) are inositol-1,4,5-trisphosphate binding proteins[37]. PRIP1 is expressed mainly in the brain, whereas PRIP2 is expressed ubiquitously[38]. PRIPs bind to GABARAP, to the intracellular domains of $GABA_A$R β subunits and, more weakly, to γ2 subunits[38,39]. These findings prompted the hypothesis that PRIPs modulate $GABA_A$Rs by competitively inhibiting GABARAP binding[39]. However, a more recent study[40] suggests that PRIPs act as bridging proteins between GABARAP and $GABA_A$Rs, facilitating the transport of γ2-containing receptors. This model was derived largely from studies of PRIP1–PRIP2 double knockout (PRIP-DKO) mice, in which the association between $GABA_A$Rs and GABARAP in neurons was significantly reduced[40]. Furthermore, PRIP-DKO mice have reduced sensitivity to diazepam, suggesting that there is an alteration in their γ2-containing $GABA_A$Rs[40]. PRIP1-knockout mice showed a similar phenotype[39]. In a complementary approach, peptides were used to disrupt the binding of PRIP1 to $GABA_A$R subunits, resulting in a reduction in cell-surface expression of γ2-containing $GABA_A$Rs in cultured cell lines and neurons[40]. Thus, PRIP and GABARAP proteins might jointly participate in the trafficking of $GABA_A$Rs to the synaptic membrane.

PRIPs might also regulate $GABA_A$R function by controlling their phosphorylation. Phosphorylation has been shown to dynamically modulate $GABA_A$R function, and β subunits are substrates for protein kinase C (PKC) and cyclic-AMP-dependent protein kinase A (PKA)[41]. Dephosphorylation of $GABA_A$Rs by protein phosphatase 1α (PP1α) terminates phosphorylation-dependent receptor modulation[42], and PP1α has been shown to be inactivated by PRIP1 (REF. 43). In one study, PRIP1-knockout mice exhibited enhanced PP1α activity, resulting in diminished phosphorylation of $GABA_A$Rs by PKA and subsequent changes in hippocampal neuronal inhibition[42]. Lastly, a recent study implicated PRIPs in the constitutive internalization of recombinant $GABA_A$Rs from the plasma membrane of non-neuronal cells[44]. Thus, PRIPs might have a central role in controlling $GABA_A$R function through at least three distinct mechanisms: the trafficking of $GABA_A$Rs, the modulation of $GABA_A$R phosphorylation and the internalization of $GABA_A$Rs.

Palmitoylation and GODZ. Palmitoylation is the covalent attachment of the saturated fatty acid palmitate to a protein. It has been shown to have a role in protein trafficking and function at both inhibitory and excitatory synapses[45]. Two groups have demonstrated that cysteine residues

Palmitoylation
The covalent attachment of a palmitate (16-carbon saturated fatty acid) molecule to a cysteine residue through a thioester bond.

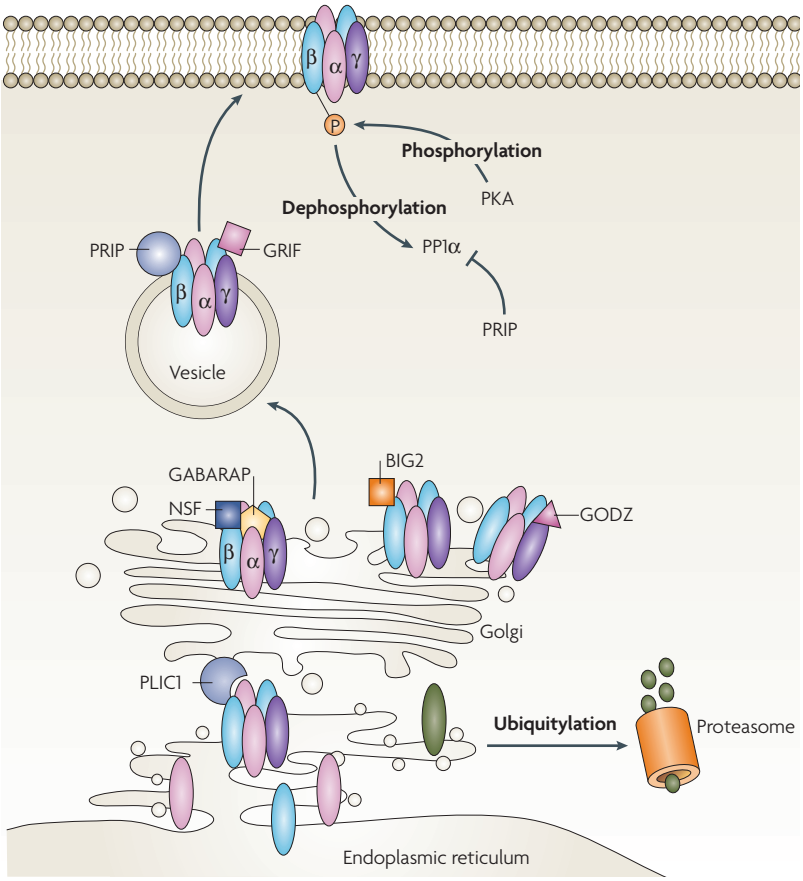

Figure 2 | **Trafficking of GABA$_A$ receptors.** GABA (γ-aminobutyric acid) type A receptor (GABA$_A$R) subunits are synthesized and assembled into pentameric structures in the endoplasmic reticulum (ER). This process is carefully regulated. The fate of GABA$_A$R subunits can be modulated by ubiquitylation and subsequent ER-associated degradation by the proteasome. Ubiquitylated GABA$_A$R subunits can also be modulated by their association with PLIC1. PLIC1 facilitates GABA$_A$R accumulation at the synapse by preventing the degradation of ubiquitylated GABA$_A$Rs. Exit into the Golgi network and subsequent trafficking to the plasma membrane are also facilitiated by a number of GABA$_A$R-associated proteins. GABA$_A$ receptor-associated protein (GABARAP) associates with the γ2 subunit of GABA$_A$Rs and aids in the trafficking of GABA$_A$Rs from the Golgi network to the plasma membrane. *N*-ethylmaleimide-sensitive factor (NSF) and brefeldin-A-inhibited GDP/GTP exchange factor 2 (BIG2) are also localized to the Golgi network, where they bind to the β subunits of GABA$_A$Rs and modulate GABA$_A$R trafficking. Palmitoylation of γ subunits occurs in the Golgi apparatus as a result of an association with the palmitoyltransferase Golgi-specific DHHC zinc-finger-domain protein (GODZ), and is a critical step in the delivery of GABA$_A$Rs to the plasma membrane. GABA$_A$R-interacting factor proteins (GRIFs) have a role in the trafficking of GABA$_A$Rs to the membrane. Phospholipase-C-related catalytically inactive proteins (PRIPs) also have essential roles in the trafficking of GABA$_A$Rs and in modulating the phosphorylation state of GABA$_A$Rs. PKA, protein kinase A; PP1α, protein phosphatase 1α. Figure modified, with permission, from REF. 146 © (2006) Springer Verlag.

in the intracellular domain of γ subunits are substrates for palmitoylation, and this modification is critical for the delivery of GABA$_A$Rs to synapses[46,47]. Golgi-specific DHHC zinc-finger-domain protein (GODZ) has been shown to mediate the palmitoyl acyl transfer to these subunits[46]. Of the 23 members of the DHHC cysteine-rich-repeat-domain (DHHC-CRD) protein family, only GODZ and its close paralogue Sertoli-cell gene with a zinc-finger domain β (SERZβ) can efficiently palmitoylate the γ2 subunit[48]. Furthermore, studies using dominant-negative GODZ or GODZ-specific RNA interference (RNAi) have demonstrated that GODZ is the principal palmitoyltransferase for GABA$_A$Rs[48]. GODZ is not found at inhibitory synapses, but it is enriched in the *trans*-Golgi network and it is essential for the accumulation of γ2-containing GABA$_A$Rs at synapses and for synaptic inhibitory function[46,48]. Therefore, GODZ presumably controls GABA$_A$R trafficking in the secretory pathway and the delivery of these receptors to the plasma membrane[46].

BIG2. Brefeldin-A-inhibited GDP/GTP exchange factor 2 (BIG2) has an important role in the vesicular trafficking of GABA$_A$Rs to the plasma membrane. A yeast two-hybrid screen showed that BIG2 can bind to the intracellular domain of the β3 subunit, and it has since been shown to have high binding affinity for the intracellular loops of all β subunits[49]. In hippocampal neurons BIG2 is largely localized to the *trans*-Golgi network, but it is also found in trafficking vesicles and at the synaptic plasma membrane[49]. BIG2 has a known role in membrane budding and vesicular transport from the Golgi apparatus[50]. Taken together, these data suggest that the main function of BIG2 is in the intracellular trafficking of GABA$_A$Rs to the plasma membrane.

GRIF/TRAK proteins. GABA$_A$R-interacting factor 1 (GRIF1; also known as TRAK2) was first described as a protein that interacts with the β2 subunit of GABA$_A$Rs[51]. It is a member of the TRAK family of coiled-coil domain proteins that have been implicated in the trafficking of intracellular vesicles. GRIF1 and TRAK1 both interact with the microtubule-associated motor protein kinesin[52,53]. TRAK1 has also been shown to interact with GABA$_A$Rs[54], suggesting a role for these proteins in regulating the motor-dependent transport of GABA$_A$Rs. Interestingly, deletion of TRAK1 in mice leads to hypertonia and reduced GABA$_A$R expression in the brain and in motor neurons[54].

Clustering GABA$_A$Rs at synapses

After navigating their way through the secretory pathway, GABA$_A$Rs are inserted into the plasma membrane, where they can access inhibitory postsynaptic specializations or extrasynaptic sites, depending on their subunit composition (FIG. 3). The mechanisms that facilitate these distinct subcellular fates are described below.

Synaptic versus extrasynaptic GABA$_A$Rs. GABA$_A$Rs on the neuronal cell surface exist as diffuse populations or as synaptic or extrasynaptic clusters. Lateral diffusion in the plasma membrane allows continual exchange between these groups[55,56]. GABA$_A$Rs that can bind bungarotoxin have been used to examine the subcellular sites of GABA$_A$R insertion into the neuronal membrane. These studies have demonstrated that most receptors are delivered to extrasynaptic locations in the plasma membrane. Over time, diffusion and trapping increase the population of synaptic receptors[57].

Heteromeric GABA$_A$Rs retain distinct cell-surface expression patterns, dependent on their subunit

RNA interference (RNAi). A molecular method in which small interfering RNA sequences are introduced into cells or tissues to decrease the expression of target genes.

Table 1 | **Summary of $GABA_A$ receptor-associated proteins**

Protein	Interacting $GABA_AR$ subunits	Subcellular localization	Putative functions	Refs
AP2	β and γ	Clathrin-coated pits	Receptor endocytosis	87,89,92,93
BIG2	β1–3	Golgi, trafficking vesicles, plasma membrane	Receptor trafficking	49
GABARAP	γ2	Mainly in Golgi	Receptor trafficking	22–24, 26–33
Gephyrin	α2	Synaptic sites	Receptor clustering and stabilization at synaptic sites	55,69–71, 75–77,79
GODZ	γ	Mainly in Golgi	Palmitoylation of γ subunits and receptor trafficking	46,48
GRIF1 and 2 (TRAK1 and 2)	β2	Intracellular compartments	Receptor trafficking	51,54
HAP1	β1–3	Endosomes	Post-endocytic sorting of $GABA_AR$s	88
NSF	β1–3	Golgi and plasma membrane	Receptor trafficking	34
PLIC1	α and β	Intracellular compartments	Modulates receptor cell-surface expression	18
PRIP1 and 2	β1–3 and γ2	Intracellular compartments	Regulation of receptor phosphorylation/trafficking	39,40,42,44
Radixin	α5	Plasma membrane	Receptor clustering and binding to actin cytoskeleton	84

AP2, clathrin-adaptor protein 2; BIG2, brefeldin-A-inhibited GDP/GTP exchange factor 2; GABA, γ-aminobutyric acid; $GABA_AR$, GABA type A receptor; GABARAP, $GABA_A$ receptor-associated protein; GODZ, Golgi-specific DHHC zinc-finger-domain protein; GRIF, $GABA_AR$-interacting factor; HAP1, Huntingtin-associated protein 1; NSF, *N*-ethylmaleimide-sensitive factor; PLIC1, protein linking IAP to the cytoskeleton; PRIP, phospholipase-C-related catalytically inactive protein.

composition. Most surface clusters of γ2-containing $GABA_AR$s are synaptic, whereas β3-containing $GABA_AR$s are more likely to be diffuse and/or extrasynaptic[55,58]. α5-containing receptor clusters are predominantly extrasynaptic[8,9]. $GABA_AR$s containing other receptor subunits, such as δ, appear as diffuse populations on the neuronal surface[59,60] and are exclusively located outside the synapse at perisynaptic and extrasynaptic locations[61]. These extrasynaptic α5- and δ-containing $GABA_AR$s are considered to be the main receptors that mediate tonic inhibition.

Gephyrin-dependent clustering of $GABA_AR$s. One protein that has been strongly implicated in regulating the clustering of $GABA_AR$s at inhibitory synapses is the multifunctional protein gephyrin, which was first identified by its association with glycine receptors[62]. Gephyrin binds directly to the intracellular domain of the β subunit of glycine receptors, stabilizing them at inhibitory synapses in the spinal cord[63–67]. Gephyrin is also widely expressed in non-neuronal tissues[65]. In the brain it is found in neurons, enriched at postsynaptic specializations that contain $GABA_AR$ subtypes composed of α (1–3), β (2 and 3) and γ2 subunits[68].

Reducing gephyrin expression compromises the accumulation of $GABA_AR$ subtypes containing α2 or γ2 subunits at inhibitory synapses[55,66,69–71], although there is no change in the overall levels of these subunits[71] and only a modest reduction in the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) or GABA-induced whole-cell currents[66]. In addition, surface clusters of $GABA_AR$s formed in the absence of gephyrin were three times more mobile than those in control neurons[55], indicating that gephyrin has a role in enhancing the confinement of $GABA_AR$s at synaptic sites. Furthermore, gene knockout of collybistin, an established binding partner for gephyrin[72,73], also leads to loss of synaptic $GABA_AR$ clusters[74]. Together, these results support the concept that gephyrin might promote the stability of α2- and/or γ2-containing $GABA_AR$ clusters.

A loss of α3 and β2 or β3 subunits was observed in spinal cord neurons of gephyrin-knockout mice, whereas there were only minimal changes in α1 or α5 subunits in hippocampal and spinal cord neurons[66,70]. These observations suggested the existence of gephyrin-dependent and -independent $GABA_AR$ clustering mechanisms. However, the development of compensatory clustering mechanisms in neurons devoid of gephyrin cannot be discounted.

The molecular mechanisms that underlie the gephyrin-dependent clustering of $GABA_AR$s remain poorly understood. Evidence suggests that a domain that is critical for clustering might exist in the γ2 subunit, as cultured neurons from γ2-knockout mice are devoid of both $GABA_AR$s and gephyrin at postsynaptic sites[69,75]. In an attempt to identify such a domain, chimeric α2/γ2 and δ/γ2 receptors have been studied[76,77]. These studies suggested that the intracellular loop and/or TM4 of the γ2 subunit are critical for $GABA_AR$ synaptic clustering[76,77]; however, whether these domains actually mediate their

Yeast two-hybrid screen
A system used to determine whether two proteins interact. It involves the expression of two proteins in yeast: the plasmids encoding these proteins are fused to the GAL4 DNA-binding and activation domains. If the proteins interact, the resulting complex drives the expression of a reporter gene, commonly β-galactosidase.

Miniature inhibitory postsynaptic current
(mIPSC). The postsynaptic current that results from the activation of synaptic receptors by neurotransmitters (GABA or glycine) that are usually released from a single vesicle.

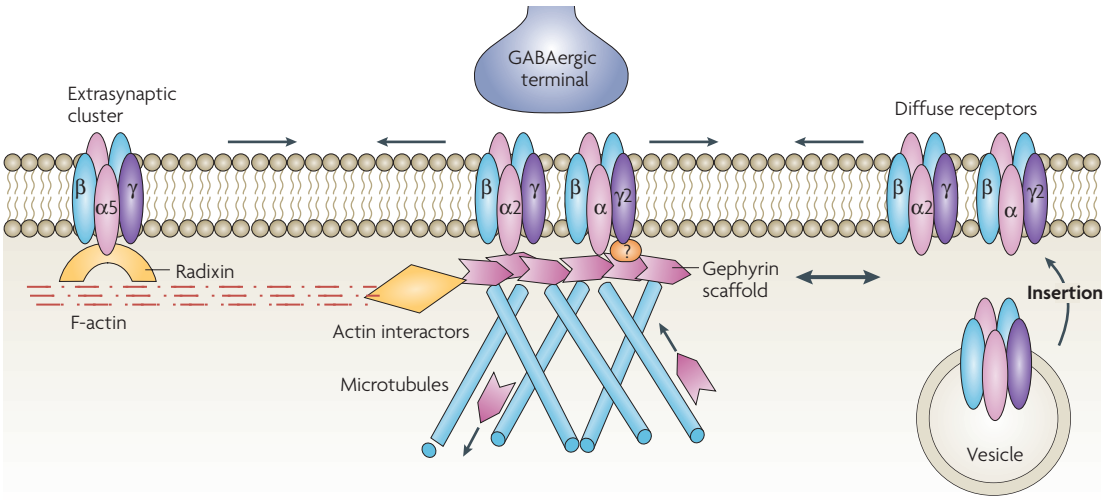

Figure 3 | **Dynamic regulation of receptor lateral mobility at the GABAergic synapse.** GABA (γ-aminobutyric acid) type A receptors ($GABA_A$Rs) are inserted into the plasma membrane at extrasynaptic sites; they can then diffuse into synaptic sites. Lateral diffusion (indicated by the horizontal single-headed arrows) in the plasma membrane allows continual exchange between diffuse receptor populations and synaptic or extrasynaptic receptor clusters, with anchoring molecules tethering or corralling moving receptors. The synaptic localization of α2-containing $GABA_A$Rs is maintained by direct binding to gephyrin, which binds to microtubules and actin interactors (such as the GDP/GTP exchange factor collybistin[72], mena/VASP (vasodilator-stimulated phosphoprotein)[147] and profilins 1 and 2 (REFS 147,148)). No direct interaction between gephyrin and the γ2 subunit has been demonstrated; however, gephyrin depletion increases γ2-containing cluster mobility, and loss of the γ2 subunit results in postsynaptic sites that are devoid of gephyrin. This suggests that there is an unidentified intermediary interactor or post-translational modification that links γ2-containing $GABA_A$Rs and gephyrin. Alternatively, clustering of γ2-containing $GABA_A$Rs might occur through a gephyrin-independent mechanism. Gephyrin also displays local lateral movements (indicated by the double-headed arrow) and removal or addition by microtubule-dependent trafficking, contributing additional mechanisms to the regulation of synaptic transmission. The extrasynaptic localization of α5-containing $GABA_A$Rs is controlled by the binding of the α5 subunit to activated radixin, which directly binds F-actin.

effects in a mechanism that is dependent on gephyrin remains to be established.

Efforts to show gephyrin binding to native $GABA_A$Rs have been unsuccessful[63]. Similarly, co-expression of gephyrin and α1–3, β1–3 and γ2 $GABA_A$R subunits in HEK-293 cells revealed only a weak interaction with the β3 subunit[78]. Interestingly, a recent study[79] identified a 10-amino-acid hydrophobic motif in the major intracellular domain of the α2 subunit that is responsible for the targeting of $GABA_A$R subunits to inhibitory synapses. Critically, this phenomenon is dependent on gephyrin expression[79]. In addition, this motif was demonstrated to mediate the direct interaction of the intracellular domain of the α2 subunit with gephyrin in *in vitro* binding assays[79]. However, under the same conditions, minimal binding of gephyrin to the intracellular domains of the γ2 and β3 subunits was evident[79]. The interaction of the α2 intracellular domain with gephyrin was blocked by low concentrations of detergent[79], thus providing a possible explanation as to why previous studies did not identify such a direct association between gephyrin and $GABA_A$Rs.

In summary, these results provide strong evidence that gephyrin can bind directly to receptor subtypes containing α2 subunits and regulate their synaptic targeting, but the relevance of this mechanism for receptor subtypes containing other α-subunit variants remains to be evaluated. Significantly, a large number of gephyrin splice variants have been identified[80], and the synaptic localization and function of gephyrin can be regulated by both activity[81,82] and phosphorylation[83]. It will therefore be of merit to examine the roles that these different variants of gephyrin have in regulating the synaptic clustering of distinct $GABA_A$R subtypes.

Gephyrin-independent clustering of $GABA_A$Rs. Gephyrin-independent $GABA_A$R clustering mechanisms are suggested by the presence of clustered receptors and mIPSCs in gephyrin-knockout mice[66,70]. Recently, radixin, an ERM (ezrin, radixin, moesin)-family protein, was identified as a specific interactor for the intracellular domain of the α5 subunit[84]. ERM proteins exist in an inactive conformation and are activated by phosphatidylinositol-4,5-bisphosphate binding and subsequent phosphorylation of the carboxyl terminus (for a review, see REF. 85). In neurons, depletion of radixin dramatically decreased α5-containing-$GABA_A$R clustering, although total cell-surface levels of the α5 subunit remained unchanged[84]. Radixin seems to directly link the α5 subunit to the actin cytoskeleton, as activated radixin can bind both the α5 subunit and F-actin[84]. The apparent radixin binding domain in the α5 subunit is a highly conserved region that is also found in α1–3 subunits, differing in only the last two amino acids in the α2 subunit. Further work is clearly needed to elucidate the mechanism of radixin-dependent $GABA_A$R anchoring.

Endocytosis and post-endocytic $GABA_AR$ sorting

$GABA_AR$ endocytosis. $GABA_ARs$ undergo extensive endocytosis in both heterologous and neuronal systems. Although a clathrin-independent endocytic pathway has been demonstrated in heterologous cells[86], clathrin-dependent endocytosis seems to be the major internalization mechanism for neuronal $GABA_ARs$[87] (FIG. 4), with approximately 25% of β3-containing cell-surface $GABA_ARs$ being internalized within 30 minutes[88]. Blocking clathrin-dependent endocytosis results in reduced $GABA_AR$ internalization[87,89,90] and a large increase in mIPSC amplitude[87], consistent with an increase in cell-surface receptor levels[87,89,90].

The clathrin-adaptor protein 2 (AP2) complex has a critical role in recruiting membrane-associated proteins into clathrin-coated pits. AP2 is composed of four distinct subunits (α, β2, μ2 and σ2; reviewed in REF. 91). $GABA_ARs$ in the brain are intimately associated with AP2 through a direct binding of the β1–3 and γ2 $GABA_AR$ subunits to the μ2 subunit of this complex[87].

In the β2 $GABA_AR$ subunit, a dileucine motif has been identified that is important for clathrin-dependent $GABA_AR$ internalization in heterologous cells[89]. In addition, an atypical AP2 binding motif in the intracellular domains of $GABA_AR$ β subunits has been identified[92]. Intriguingly, this binding motif contains the major sites of phosphorylation for PKA and PKC, and phosphorylation of these sites reduces binding to the μ2 subunit of AP2 (REF. 92). A peptide corresponding to the AP2 binding motif in the β3 subunit binds to AP2 with high affinity only when it is dephosphorylated[92]. Furthermore, this peptide enhanced mIPSC amplitude and whole-cell $GABA_AR$ currents.

More recently, another AP2 binding motif, centred around tyrosines 365 and 367 in the $GABA_AR$ γ2 subunit, has been identified[93]. These tyrosine residues are the principal sites for phosphorylation by Src kinase[94]. A peptide containing residues Y365 and Y367 exhibits high affinity for the μ2 subunit, and the affinity of this interaction is dramatically decreased by phosphorylation of these sites[93]. Introduction of the non-phosphorylated γ2 peptide into neurons produced a large increase in the mIPSC amplitude and increased the number of cell-surface $GABA_ARs$. Intriguingly, co-dialysis of neurons with both the non-phosphorylated β3- and the γ2-subunit peptides produced an additive effect on mIPSC amplitudes[93].

Together, these results provide direct evidence that phosphorylation of $GABA_AR$ subunits at distinct AP2 binding sites can regulate the cell-surface stability of $GABA_ARs$ and the strength of synaptic inhibition. Moreover, they also provide a mechanism by which neurotransmitter and/or growth factor signalling pathways that regulate the activity of protein kinases and phosphatases[41,95–97] could influence the efficacy of synaptic inhibition by controlling the stoichiometry of $GABA_AR$ phosphorylation and, thus, $GABA_AR$ endocytosis.

$GABA_AR$ recycling and lysosomal degradation. Once they have been endocytosed, most internalized $GABA_ARs$ recycle back to the plasma membrane over short time frames; however, over longer time periods they are targeted for lysosomal degradation[88]. Clearly the fate of internalized $GABA_ARs$ therefore has a critical role in controlling cell-surface receptor levels and hence the efficacy of synaptic inhibition. Huntingtin-associated protein 1 (HAP1)[98] is a $GABA_AR$-associated protein that binds the intracellular loop of β subunits *in vitro* and *in vivo*[88]. HAP1 is a cytoplasmic protein with several central coil-coiled domains that are likely to regulate protein–protein interactions. Overexpression of HAP1 in neurons inhibits $GABA_AR$ degradation and consequently increases receptor recycling[88]. Furthermore, HAP1 overexpression increased steady-state surface levels of $GABA_ARs$ and produced a 63% increase in mIPSC amplitude, showing that increased surface receptor levels have a dramatic functional effect[88]. The mechanism that underlies post-endocytic $GABA_AR$ sorting remains to be elucidated, and HAP1's specific role in this process is also an area of active research. The impact of HAP1 regulation of $GABA_ARs$ was recently shown in the hypothalamus, where downregulation of HAP1 resulted in decreased $GABA_AR$ levels, causing decreased food intake and weight loss[99]. An unresolved issue is whether HAP1 promotes recycling of $GABA_ARs$ or prevents their lysosomal degradation.

Compromised $GABA_AR$ trafficking in disease

The significance of the aforementioned mechanisms for maintaining homeostatic synaptic inhibition is highlighted by the multiple neurological and psychiatric diseases in which $GABA_AR$ dysfunction has been implicated. These include epilepsy[100], anxiety disorders[2], Huntington's disease[101], Angelman syndrome[102], fragile X syndrome[103], schizophrenia[104] and drug abuse[105]. In this section, we highlight recent findings related to a few of these disorders.

Epilepsy. The epileptic state represents a dramatic change in balance between excitatory and inhibitory activity. Studies have shown that seizure activity results in altered $GABA_AR$ trafficking and/or subunit expression in animal models of *status epilepticus* (SE) and temporal lobe epilepsy (TLE), as well as in patients[100,106]. These changes involve both up- and downregulation, depending on the particular $GABA_AR$ subunit in question and the stage of evolution of the seizure state that is being studied.

SE is a life-threatening state in which seizures occur unremittingly[107]. Decreases in synaptic $GABA_ARs$, resulting from enhanced endocytosis, have been observed in animals in which SE has been experimentally induced[108–110]. The loss of these synaptic receptor populations, which are normally benzodiazepine-sensitive, might explain the rapid development of pharmacoresistance in patients with SE, and might also explain the non-terminating nature of the seizures. A recent study showed that there is decreased phosphorylation of β3 $GABA_AR$ subunits during SE, with a resulting increased association of the receptors with the clathrin adaptor AP2 (REF. 110) (FIG. 5a). Enhancing $GABA_AR$ subunit phosphorylation or selectively blocking subunit binding to AP2 increased $GABA_AR$ surface expression levels and normalized synaptic inhibition in hippocampal slices derived from

Clathrin
One of the main protein components of the coat that is formed during membrane endocytosis.

Clathrin-adaptor protein 2 (AP2) complex
A heterotetrameric complex composed of subunits called adaptins that have an important role in clathrin-dependent membrane endocytosis.

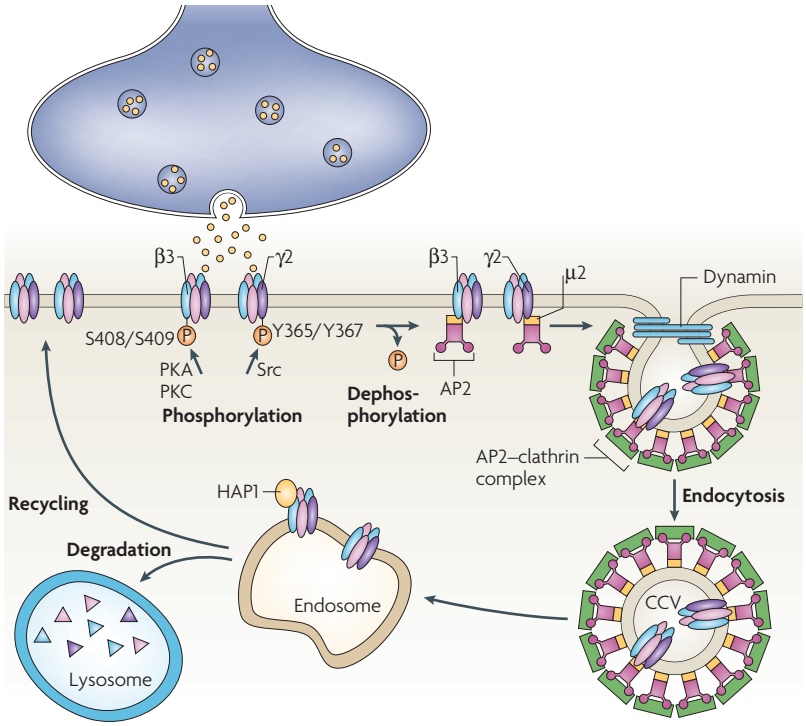

Figure 4 | **Regulation of $GABA_A$ receptor endocytosis and post-endocytic sorting.** Clathrin-dependent endocytosis is the major internalization mechanism for neuronal GABA (γ-aminobutyric acid) type A receptors ($GABA_A$Rs). The intracellular loops of the $GABA_A$R β and γ subunits interact with the clathrin-adaptor protein 2 (AP2) complex. Binding of the μ2 subunit of AP2 is inhibited by phosphorylation of the AP2-interacting motifs in the $GABA_A$R subunits, increasing cell-surface receptor levels and enhancing the efficacy of inhibitory synaptic transmission. Once the $GABA_A$Rs have been endocytosed in clathrin-coated vesicles (CCVs), the vesicles uncoat and fuse with early or sorting endosomes, resulting in the $GABA_A$Rs being subsequently recycled to the plasma membrane or degraded in lysosomes. Huntingtin-associated protein 1 (HAP1) interacts with the β subunits and promotes receptor recycling to the plasma membrane. Protein kinase A (PKA) and protein kinase C (PKC) regulate the phosphorylation of S408 and S409 in the AP2-binding motif of β3 subunits, whereas Y365 and Y367 in the γ2 subunit are phosphorylated by Src kinase. Figure modified, with permission, from REF. 146 © (2006) Springer Verlag.

mice with SE[110]. Thus, novel therapeutic strategies for SE might one day be based on preventing or reversing this aberrant internalization of $GABA_A$Rs.

Altered $GABA_A$R expression has also been observed in animal models of TLE; however, these models have generally shown increases in the expression of synaptic $GABA_A$Rs, at least in the dentate gyrus[111]. Corresponding increases in the expression of $GABA_A$R-associated proteins, such as gephyrin, and in the size and density of postsynaptic $GABA_A$R clusters have also been demonstrated[112]. This suggests that novel GABAergic synapses form, perhaps as a result of the aberrant sprouting of GABAergic axons[106]. Extrasynaptic $GABA_A$R subunits (α4 and δ) have also been reported to be increased in granule cells of the dentate gyrus in rat models of TLE[113–115]; however, one study in mice reported decreases in δ-subunit expression in these cells[116]. Analyses of hippocampal tissue from patients with TLE reveal alterations in $GABA_A$R subunit expression patterns that are similar to those that are observed experimentally[117,118].

GABAergic plasticity
Changes in local activity that lead to longer-term increases or decreases in inhibitory synaptic strength.

Human genetic studies have provided further evidence that abnormal $GABA_A$R function contributes to epilepsy disorders. Multiple distinct mutations in the γ2 (REFS 119–121), α1 (REFS 122,123) and δ subunits[124] have been identified in patients with epilepsy. Although the exact mechanism by which each of these mutations contributes to seizure disorders remains to be fully elucidated, deficits in the assembly, trafficking and function of recombinant mutant receptors have been described[119,120,125–127]. For example, a missense mutation in the α1 subunit (A322D) leads to subunit retention in the ER, followed by ubiquitin-dependent degradation[128]. This results in lower overall levels of α1-containing $GABA_A$Rs at the cell surface.

Drug abuse. There is considerable evidence supporting a role for $GABA_A$Rs in mediating the addictive properties of drugs of abuse[105,129]. In particular, chronic use of alcohol or benzodiazepines, both of which are allosteric modulators of $GABA_A$Rs, can lead to drug tolerance, dependence and withdrawal symptoms following drug cessation. Changes in the mRNA and protein expression of various $GABA_A$R subunits have been documented after alcohol and benzodiazepine administration in both cultured neurons and animal models[130,131]. However, the mechanisms responsible for these alterations have only recently begun to be elucidated. Significant alterations in the surface expression and composition of both synaptic and extrasynaptic $GABA_A$R populations have been observed after a single intoxicating dose of alcohol in rats[132] (FIG. 5b). These changes were found to be persistent after chronic alcohol administration and subsequent withdrawal[132,133]. This long-term plasticity in $GABA_A$Rs is likely to involve changes in the phosphorylation of $GABA_A$R subunits and alterations in the endocytosis of specific $GABA_A$R subtypes. For example, the association of PKC with $GABA_A$R subunits is altered after chronic ethanol exposure[134]. Increased associations between clathrin adaptor proteins and α1 subunits have also been demonstrated[135], suggesting that enhanced clathrin-mediated endocytosis of α1-containing $GABA_A$Rs contributes to changes in $GABA_A$R trafficking after chronic alcohol use (FIG. 5b). Interestingly, the well-documented phenomenon of cross-tolerance to benzodiazepines after chronic alcohol use[136] suggests that similar mechanisms might be responsible for tolerance to both of these drugs. Thus, understanding tolerance-inducing alterations in $GABA_A$R trafficking should not only advance our understanding of the disease process that leads to alcoholism but should also improve the development of drugs to treat insomnia and anxiety disorders without causing tolerance.

Schizophrenia. Altered expression of several proteins that are involved in GABAergic transmission has been reported in studies of post-mortem tissue from subjects with schizophrenia. Significant reductions in the mRNA levels of glutamic acid decarboxylase 67 (GAD67), one of the major GABA synthesizing enzymes, and the GABA membrane transporter GAT1 have been observed in a subpopulation of interneurons in the prefrontal cortex of

schizophrenic subjects[137,138]. In addition, a compensatory upregulation of α2-containing $GABA_A$Rs in the axon initial segment of pyramidal neurons has been demonstrated[137]. Reduced GABAergic signalling between these affected interneurons and pyramidal cells has been postulated to contribute to cognitive deficits associated with schizophrenia[104].

In vivo analysis of animal models will help to determine the extent to which aberrant GABAergic plasticity contributes to the pathophysiology of schizophrenia. For example, mice lacking the α3 $GABA_A$R subunit showed select deficits in pre-pulse inhibition (PPI), which could be normalized by treatment with the antipsychotic drug haloperidol[139]. Deficits in PPI have been associated with a number of psychiatric disorders, including schizophrenia, and are a measure of a diminished ability to process sensorimotor information[140]. There was a dramatic loss of synaptic $GABA_A$Rs and gephyrin clusters in the thalamic reticular nucleus[141] (one of the main regions in the brain where the α3 subunit is normally expressed[142]) of α3-subunit-knockout mice and a resultant absence of functional inhibitory

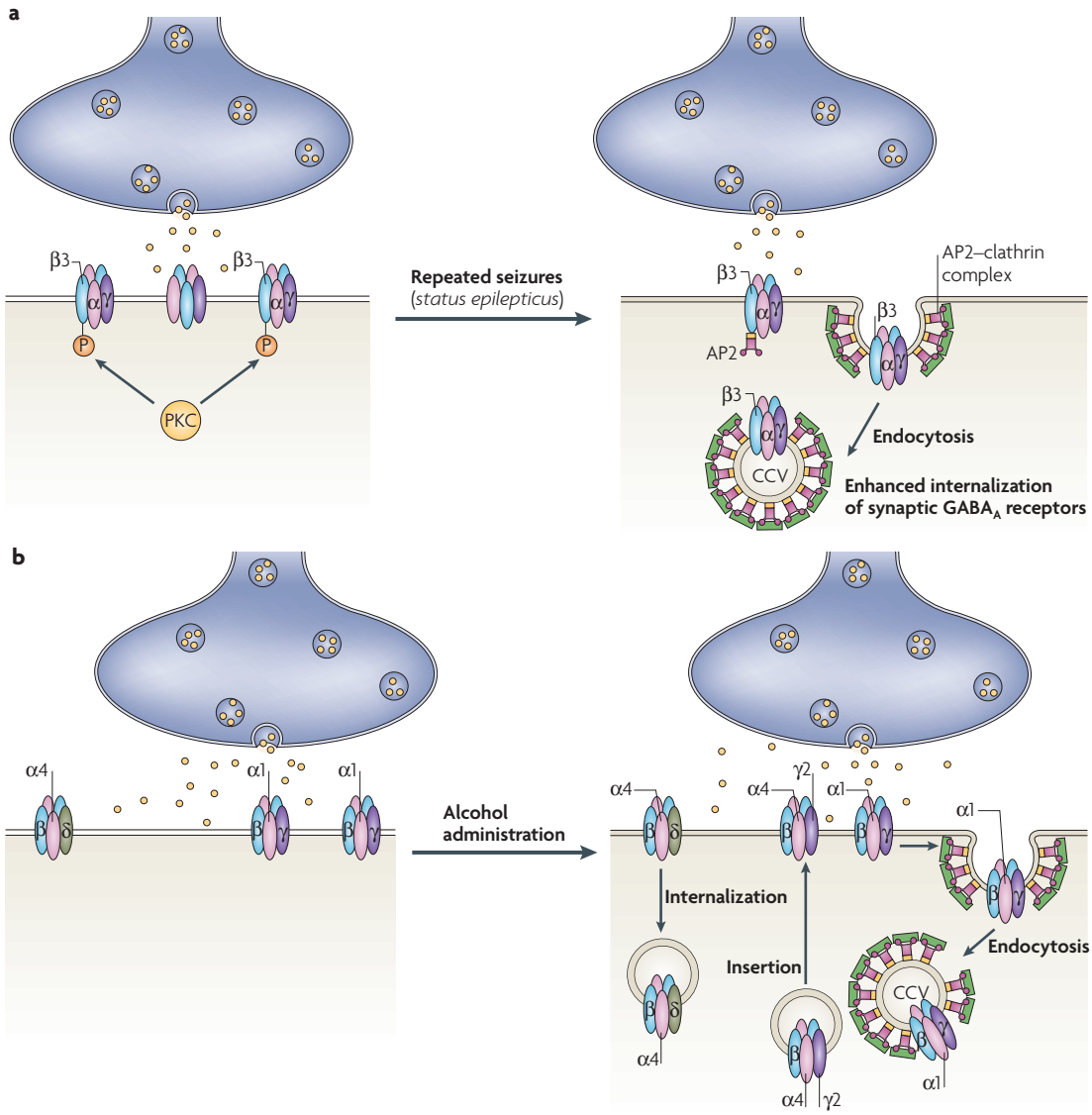

Figure 5 | **Dysregulation of $GABA_A$ receptor trafficking in neurological disease. a** | Repetitive, non-abating seizures that lead to *status epilepticus* result in a decrease in the phosphorylation of GABA (γ-aminobutyric acid) type A receptor ($GABA_A$R) β3 subunits by protein kinase C (PKC). This leads to an increased association with the clathrin-adaptor protein 2 (AP2) complex, followed by increased internalization through clathrin-mediated endocytosis. Decreased numbers of synaptic $GABA_A$Rs lead to reduced synaptic inhibition (that is, increased excitatory drive and a lower seizure threshold) as well as decreased benzodiazepine sensitivity. **b** | Alcohol-induced plasticity in $GABA_A$Rs involves changes in both synaptic and extrasynaptic $GABA_A$R populations. After alcohol administration there is increased internalization of δ-containing extrasynaptic $GABA_A$Rs. There is also increased clathrin-dependent internalization of α1-containing synaptic $GABA_A$Rs. Insertion of distinct $GABA_A$R populations (α4βγ2-containing populations) at synaptic sites has been proposed to serve a compensatory role at inhibitory synapses; however, these receptors differ from normal synaptic $GABA_A$Rs in their physiological functions and are benzodiazepine-insensitive.

receptors throughout development in this critical brain region. Deficits in PPI have also been observed in mutant mice in which there is a selective reduction in hippocampal α5-containing $GABA_A$Rs[143]. Together, these findings suggest that sensorimotor gating is highly sensitive to an imbalance in inhibitory neurotransmission, and that hypofunction of select $GABA_A$R populations can lead to a schizophrenia-related cognitive impairment. Pharmacological interventions to increase $GABA_A$R function and/or trafficking of relevant $GABA_A$R subpopulations might help to alleviate some of the symptoms of schizophrenia and other psychiatric disorders.

Conclusions and outlook

Fast inhibitory GABAergic synaptic transmission is a principal determinant of neuronal excitability. It is dependent on the delivery of individual $GABA_A$R subtypes, which are endowed with unique physiological and pharmacological properties, to their appropriate synaptic or extrasynaptic sites, where they mediate phasic and tonic inhibition, respectively.

The synthesis and assembly of $GABA_A$Rs in the ER is an important control point in the determination of receptor diversity on the plasma membrane. Results from knockout mice have illustrated that there are preferential receptor-subunit partnerships, but how these preferences are orchestrated remains to be determined. It is becoming apparent that subunits in the ER are subject to activity-dependent ubiquitylation, which decreases their stability and half-life and limits the rate of insertion of newly synthesized receptors into the plasma membrane. It will be exciting to determine whether the various $GABA_A$R subunits are differentially ubiquitylated, as this would allow neuronal activity to shape the levels and pharmacological properties of $GABA_A$Rs on target cells. Modulating receptor palmitoylation or binding to accessory proteins during their passage through the Golgi apparatus might further refine our understanding of how these processes shape the diversity of $GABA_A$Rs on the plasma membrane.

$GABA_A$Rs exhibit high rates of diffusion at the cell surface, facilitating their delivery to synaptic sites or their entry into coated pits for removal by clathrin-dependent endocytosis. It is becoming clear that endocytosis is regulated by phosphorylation-dependent mechanisms; more specifically, receptor binding to clathrin-associated proteins can be negatively modulated by the phosphorylation of serine or tyrosine residues in specific $GABA_A$R subunits. This could allow cell-signalling pathways that regulate $GABA_A$R phosphorylation to also influence $GABA_A$R cell-surface stability. Determination of the relevance of these processes awaits the development of knock-in mouse lines in which the phosphorylatable residues in individual AP2 binding motifs have been ablated. However, it is interesting to note that dephosphorylation of $GABA_A$Rs and their enhanced endocytosis might be responsible for the compromised synaptic inhibition that occurs during SE. Furthermore, the fate of endocytosed receptors is another determinant of steady-state cell-surface expression levels. However, our understanding of processes that control the recycling and lysosomal degradation of $GABA_A$Rs remains rudimentary.

Stabilization of $GABA_A$Rs on the plasma membrane is likely to be facilitated by multiple mechanisms. Extrasynaptic receptors mediate tonic inhibition, and the stabilization of α5-containing receptors at extrasynaptic specializations is facilitated by the actin binding protein radixin. For synaptic receptors, the multifunctional protein gephyrin is strongly implicated in stabilizing receptors that contain α2 and γ2 subunits. There is also evidence that α1-containing $GABA_A$Rs, although tightly colocalized with gephyrin, can be maintained at synaptic sites in the absence of gephyrin. Therefore, further studies are required to address the range of $GABA_A$R subunits that can bind to specific gephyrin splice variants, and the roles that these binding motifs have in the accumulation of individual subtypes at inhibitory synapses.

Resolution of these issues will provide key insights into what controls inhibitory synaptic strength and how alterations in these processes result in the development of CNS pathologies, ranging from epilepsy to schizophrenia. This information is also likely to lead to the identification of novel therapeutic drug targets that will allow the pharmacological modulation of individual $GABA_A$R subtypes.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
BIG2 | calnexin | GABARAP | GAD67 | GAT1 | gephyrin | GODZ | GRIF1 | HAP1 | NSF | PLIC1 | PLIC2 | PRIP1 | PRIP2 | radixin | TRAK1
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
Angelman syndrome | fragile X syndrome | Huntington's disease

FURTHER INFORMATION

Stephen Moss's homepage: www.med.upenn.edu/mosslab/

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