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

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Abstract

γ-Aminobutyric acid type A ($GABA_A$) receptors mediate the majority of fast synaptic inhibition in the mammalian brain, controlling activity both at the network and cellular level. The diverse functions of GABA in the central nervous system are matched not just by the heterogeneity of $GABA_A$ receptors, but also the complex trafficking mechanisms and protein-protein interactions that generate and maintain appropriate receptor cell surface localization. In this review, we discuss recent progress in our understanding of the dynamic regulation of $GABA_A$ receptor 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 $GABA_A$ receptor trafficking occur.

1. Introduction

Synaptic inhibition in the brain is largely mediated via γ-aminobutyric acid (GABA). The fast inhibitory actions of GABA are mediated via the activation of $GABA_A$ receptors ($GABA_A$Rs) in the brain[1,2] and via $GABA_C$ receptors in the retina[3], whereas the slow prolonged actions of this transmitter are mediated via 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 comprehending the cellular mechanisms that regulate their accumulation on the neuronal plasma membrane.

Molecular studies have demonstrated that $GABA_A$Rs are members of a ligand-gated ion channel superfamily, whose members include nicotinic acetylcholine, glycine and 5-hydroxy-tryptamine 3 ($5\text{-}HT_3$) receptors[6,7]. Members of this superfamily are heteropentamers that are assembled from a range of homologous subunits that share a common structure: a large N-terminal extracellular domain and 4 transmembrane domains (TMs) with a large intracellular domain between TM 3 and 4 (Fig. 1A). To date, 18 $GABA_A$R subunits have been identified. Based on sequence homology, these are divided into 7 subunit classes, each of which has multiple members; α(1–6), β(1–3), γ(1–3), δ, ε(1–3), θ, π. $GABA_A$R structural diversity is further increased via the alterative splicing of some receptor mRNAs. However, most $GABA_A$Rs are composed of 2α, 2β and 1γ (or 1δ) subunit[2] (Fig. 1B). $GABA_A$Rs with different subunit composition have different physiological and pharmacological properties, are differentially expressed throughout the brain, and targeted to different subcellular regions. For instance, receptors composed of α(1,2,3 or 5) subunits together with β and γ subunits form

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benzodiazepine-sensitive receptors that are largely synaptically located and mediate the majority of phasic inhibition in the brain[2] (with the notable exception of extrasynaptically-localized α5-containing receptors[8,9]) (Fig. 1C). In contrast, those composed of α(4/6)βδ subunits form a specialized population of predominantly extrasynaptic receptor subtypes that mediate tonic inhibition and are insensitive to benzodiazepine modulation[9]. In addition, $GABA_A$Rs at presynaptic sites also exist[10].

Here we will 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.

2. Controlling $GABA_A$R assembly

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

Limiting diversity via 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. Most studies agree that most $GABA_A$Rs expressed on the surface of neurons are composed of 2α, 2β, and 1γ subunit (the γ subunit can be replaced by δ, ε or π depending on the neuron type and 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[12]). Thus, the expression and assembly of these subunits must be carefully regulated in the ER, via mechanisms that involve classical ER-resident chaperones, such as heavy chain binding protein and calnexin[13].

Sequences in the N-terminus of $GABA_A$R subunits control receptor oligomerization, thus promoting the assembly of particular subunit combinations[12]. Oligomerization of individual $GABA_A$ receptor subunits into heteromers occurs within 5 minutes after translation[14]. However, it is inefficient, with less than 25% of translated subunits being assembled into heteromeric receptors[14]. $GABA_A$R subunit-deficient mice have provided insights into the preferential assembly of select $GABA_A$Rs *in vivo*. For example, a loss of the δ subunit from the plasma membrane of cerebellar granule cells is observed in α6 knock-out mice[15]. Similarly, there is a decrease in the α4 subunit levels in the forebrain of δ subunit-deficient mice, whereas the levels of α1 subunits 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 γ2 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 $GABA_A$R ubiquitination

The ER is responsible for the retention and degradation of misfolded or unassembled subunits and, accordingly, homomeric unassembled $GABA_A$Rs subunits have been shown to be degraded in this organelle[14,18]. ER-associated degradation (ERAD) involves protein ubiquitination and degradation via the ubiquitin-proteasome system (UPS)[19]. $GABA_A$R subunits have recently been shown to be ubiquitinated in an activity-dependent manner[20]. Chronic blockade of neuronal activity dramatically increased the levels of $GABA_A$R

ubiquitination within the ER, resulting in decreased insertion at the plasma membrane[20]. Correspondingly, increasing the level of neuronal activity resulted in a decrease in the level of $GABA_AR$ ubiquitination and an enhancement of receptor cell surface expression[20]. Thus, neuronal activity can regulate the ubiquitination of $GABA_ARs$ in the ER, affecting their rate of degradation via the UPS. This may be one mechanism neurons use to homeostatically regulate synaptic inhibition.

The fate of ubiquitinated $GABA_ARs$ is also likely to be modulated via their association with the ubiquitin-like proteins Plic-1 and Plic-2[18], which have been demonstrated to block the degradation of ubiquitinated substrates[21]. Plic-1 binds to the intracellular domain of α and β $GABA_AR$ subunits via its ubiquitin-associated (UBA) domain[18]. Plic-1 increases the half-life of $GABA_ARs$, resulting in an increase in the number of receptors available for insertion into the plasma membrane[18]. Plic-1 does not affect the rate of receptor endocytosis[18], thus it appears to function solely in the secretory pathway by stabilizing and/or inhibiting the degradation of $GABA_ARs$ by the UPS, thereby facilitating receptor accumulation at inhibitory synapses.

3. Facilitating $GABA_AR$ trafficking

After assembly in the ER, transport-competent $GABA_ARs$ 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), which are described in the following sections.

GABARAP and NSF

GABA receptor-associated protein (GABARAP) interacts with the intracellular domain of $GABA_AR$ γ subunits *in vitro* and *in vivo*[22]. It also binds to microtubules[23] and to *N*-ethylmaleimide-sensitive factor (NSF)[24], a protein involved in intracellular vesicular fusion events[25]. GABARAP is concentrated in the Golgi apparatus and in intracellular vesicles, but is not present at GABAergic synapses[22,24,26], suggesting that its main role is in the intracellular transport of $GABA_ARs$. Overexpressing GABARAP with $GABA_ARs$ results in increased cell surface receptor expression, possibly as a result of enhanced intracellular receptor trafficking[27,28,29]. This effect can be abolished by a mutation that disrupts the addition of phospholipids to GABARAP[30], which apparently increases its membrane association, and is thus critical for GABARAP to control $GABA_AR$ trafficking[30]. Analysis of GABARAP knock-out mice did not reveal any alterations in synaptic γ2-containing $GABA_AR$ numbers[31]; however, this may reflect redundancy, given the existence of other GABARAP homologs that can interact with $GABA_ARs$[32]. Recently, it was demonstrated that GABARAP is necessary for increasing cell surface $GABA_AR$ expression after *N*-methyl-D-aspartate (NMDA) receptor activation[33], suggesting that it may have a role in the regulated delivery of $GABA_ARs$ to the surface after activity, rather than in the maintenance of basal receptor levels.

NSF has also been found to bind directly to $GABA_AR$ β subunits[34]. NSF and GABARAP may act together to promote the forward trafficking of $GABA_ARs$ from the Golgi apparatus. Indeed, the subcellular distribution of both $GABA_ARs$ and NSF is disturbed when the lipid modification of GABARAP is prevented in neurons, resulting in less $GABA_ARs$ being trafficked to the plasma membrane[30]. However, another study found that overexpression of NSF significantly reduced $GABA_AR$ cell surface numbers in both heterologous systems and in neurons[34]. This is opposite to the effect observed on $GABA_ARs$ when GABARAP is overexpressed[27,28,29], and is also opposite to NSF's role in enhancing α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor surface expression[35,36]. This may

indicate that NSF has additional functions in the endocytic pathway, however further studies are required to understand the exact mechanism of how NSF regulates $GABA_AR$ numbers.

PRIP proteins

Phospholipase C-related catalytically inactive proteins (PRIP) are inositol 1,4,5-trisphosphate binding proteins[37]. PRIP-1 is expressed mainly in the brain, whereas PRIP-2 is expressed ubiquitously[38]. PRIP proteins bind to GABARAP as well as to the intracellular domains of $GABA_AR$ β subunits, and more weakly, γ2 subunits[38,39]. These findings prompted the hypothesis that PRIP proteins modulate $GABA_ARs$ by competitively inhibiting GABARAP binding[39]. However, a more recent study[40] suggests that PRIP molecules act as bridging proteins between GABARAP and $GABA_ARs$, facilitating the transport of γ2-containing receptors. This model was derived largely from studies of PRIP-1 and PRIP-2 double knock-out (PRIP-DKO) mice, in which the association between $GABA_ARs$ and GABARAP in neurons was significantly reduced[40]. Furthermore, PRIP-DKO mice have reduced sensitivity to diazepam, suggesting an alteration in γ2-containing $GABA_ARs$[40]. PRIP-1 knockout mice showed a similar phenotype[39]. In a complementary approach, peptides were used to disrupt the binding of PRIP-1 to $GABA_AR$ subunits, resulting in a reduction in cell surface expression of γ2-containing $GABA_ARs$ in cultured cell lines and neurons[40]. Thus, PRIP and GABARAP proteins may participate together in the trafficking of $GABA_ARs$ to the synaptic membrane.

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

Palmitoylation and GODZ

Palmitoylation involves the covalent attachment of the saturated fatty acid palmitate to a protein and has been shown to play a role in protein trafficking and function at both inhibitory and excitatory synapses[45]. Two groups have demonstrated that cysteine residues located within the intracellular domain of γ subunits are substrates for palmitoylation and that this modification is critical for the delivery of $GABA_ARs$ to synapses[46,47]. The Golgi-specific DHHC zinc finger domain protein (GODZ) has been shown to mediate the palmitoyl acyl transfer to these subunits[46]. Of the twenty-three members of the Asp, His, His, Cys-cysteine-rich repeat domain (DHHC-CRD) protein family only GODZ and its close paralog Sertoli cell gene with a zinc finger domain-β (Serz-β) can efficiently palmitoylate the γ2 subunit[48]. Furthermore, studies using dominant-negative GODZ or GODZ-specific RNA interferance (RNAi) have demonstrated that GODZ is the principal palmitolytransferase for $GABA_ARs$[48]. GODZ is not found at inhibitory synapses, but is enriched in the *trans*-Golgi network, and is essential for the accumulation of γ2-containing $GABA_ARs$ at synapses and for synaptic inhibitory function[46,48]. Therefore, GODZ presumably acts to control $GABA_AR$ 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) plays an important role in the vesicular trafficking of $GABA_A$Rs to the plasma membrane. A yeast two hybrid screen showed that it 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, this suggests that the main function of BIG2 is in the intracellular trafficking of $GABA_A$Rs through the exocytic pathway to the plasma membrane.

GRIF/TRAK proteins

$GABA_A$R-interacting factor 1 (GRIF-1) 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. GRIF-1 (also known as TRAK2) 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 motor neurons[54].

4. 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 are able to access inhibitory postsynaptic specializations or extrasynaptic sites, depending on subunit composition (Fig. 3). The mechanisms that facilitate these distinct subcellular fates are described below.

Synaptic vs. extrasynaptic $GABA_A$Rs

$GABA_A$Rs on the neuronal cell surface exist as diffuse receptor populations or synaptic or extrasynaptic clusters. Lateral diffusion within the plasma membrane allows for continual exchange between these receptor populations[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 increases the population of synaptic receptors[57].

Heteromeric $GABA_A$Rs retain distinct cell surface expression patterns dependent on subunit composition. Most surface receptor clusters of γ2 receptor subunits are synaptic, whereas β3-containing $GABA_A$Rs have a higher proportion of diffuse and/or extrasynaptic receptors[55,58]. α5-containing receptors are predominantly extrasynaptic[8,9]. 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[57,61]. These extrasynaptic α5- and δ- containing $GABA_A$Rs are considered the main receptors mediating tonic inhibition.

Gephyrin-dependent clustering of $GABA_A$Rs

One protein strongly implicated in regulating the clustering of $GABA_A$Rs at inhibitory synapses is the multifunctional protein gephyrin, which was first identified by its association with glycine receptors[62]. Gephyrin binds directly with the intracellular domain of the β subunit of glycine receptors, which stabilizes these proteins 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, and is enriched at postsynaptic specializations that contain $GABA_AR$ subtypes composed of α(1–3), β(2,3) 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 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 $GABA_ARs$ formed in the absence of gephyrin were three times more mobile than those in control neurons[55], indicating that gephyrin plays a role in enhancing the confinement of $GABA_ARs$ 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 may act to promote the stability of $GABA_AR$ clusters containing α2/γ2 subunits.

A loss of α3 and β(2/3) subunits were 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 underlying gephyrin-dependent clustering of $GABA_ARs$ remain poorly understood. Evidence suggests that a domain critical for clustering may exist within the γ2subunit, as cultured neurons from γ2 knock-out mice are devoid of both $GABA_ARs$ and gephyrin at postsynaptic sites[69,75]. In order to identify such a domain, chimeric α2/γ2 and δ/γ2 receptors have been studied[76,77]. These suggest that the intracellular loop and/or transmembrane domain 4 of the γ2 subunit are critical for $GABA_AR$ synaptic clustering[76,77], but whether these domains actually mediate their effects in a mechanism dependent upon gephyrin remains to be established.

Efforts to show gephyrin binding to native $GABA_ARs$ have been unsuccessful[63]. Similarly, co-expression of gephyrin and α1–3, β1–3 and γ2 $GABA_AR$ 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 within the major intracellular domain of the α2 subunit that is responsible for the targeting of $GABA_AR$ subunits to inhibitory synapses. Critically, this phenomenon is dependent upon 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 had not identified such a direct association between gephyrin and $GABA_ARs$.

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 differing variants of gephyrin play in regulating the synaptic clustering of distinct $GABA_AR$ subtypes.

Gephyrin-independent clustering of $GABA_ARs$

Gephyrin-independent $GABA_AR$ clustering mechanisms are suggested by the presence of clustered receptors and mIPSCs in gephyrin knockout mice[66,70,83]. Recently, radixin, an

ERM (ezrin, radixin, moesin)-family member protein, has been 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 (PIP_2) binding and subsequent phosphorylation of the C-terminus (for a review, see[85]). In neurons, depletion of radixin dramatically decreased α5-containing $GABA_AR$ clustering, although total cell surface levels of the α5 subunit remained unchanged[84]. Radixin appears to directly link the α5 subunit to the actin cytoskeleton, as activated radixin is capable of binding both the α5 subunit and F-actin[84]. The apparent radixin binding domain within the α5 subunit is a highly conserved region that is also found in α1–3 subunits, differing only in the last two amino acids in the α2 subunit. Further work clearly remains in elucidating the mechanism of radixin-dependent $GABA_AR$ anchoring.

5. Endocytosis and post-endocytic sorting of $GABA_ARs$

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

Within 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 within 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[92]. A peptide corresponding to the AP2 binding motif in the β3 subunit binds to AP2 with high affinity only when dephosphorylated[92]. Furthermore, this peptide enhanced mIPSC amplitude and whole cell $GABA_AR$ currents.

More recently, another AP2 binding motif, centered around tyrosines 365/7 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 Y365/7 exhibits very high affinity for the μ2 subunit, and the affinity of this interaction is dramatically decreased via phosphorylation of these sites[93]. Introduction of the non-phosphorylated γ2 peptide into neurons produced a large increase in the mIPSC amplitude, and was observed to increase the number of cell surface $GABA_ARs$. Intriguingly, co-dialysis of neurons with both the non-phosphorylated β3 and 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 signaling pathways that regulate the activity of protein kinases/phosphatases[41,95–97]; could influence the efficacy of synaptic inhibition by controlling the stoichiometry of $GABA_AR$ phosphorylation and, thus, their endocytosis.

$GABA_AR$ recycling and lysosomal degradation

Once 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 $GABA_ARs$ will therefore play 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 a dramatic functional effect of increased surface receptor number[88]. The mechanism underlying 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 down-regulation of HAP1 levels resulted in decreased $GABA_AR$ levels, causing decreased food intake and loss of body weight[99]. An unresolved issue is whether HAP1 acts to promote recycling of $GABA_ARs$ or prevents their lysosomal degradation.

6. 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 suggested. 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 down-regulation, depending on the particular $GABA_AR$ subunit in question, as well as on the time-point studied in relation to the evolution of the chronic seizure state.

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, may explain the rapid development of pharmacoresistance in patients with SE and also explain the non-terminating nature of these seizures. A recent study showed decreased phosphorylation of β3 $GABA_AR$ subunits during SE, resulting in an increased association of receptors with the clathrin adaptor AP2[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 mice with SE[110]. Thus, novel therapeutic strategies for SE may one day be based on preventing or reversing this aberrant internalization of $GABA_ARs$.

Altered $GABA_AR$ expression has also been observed in animal models of TLE, however these have generally shown increases in the expression of synaptic $GABA_ARs$, at least in the dentate gyrus[111]. Corresponding increases in the expression of $GABA_AR$-associated proteins, such as gephyrin, and in the size and density of postsynaptic $GABA_AR$ clusters have also been

demonstrated[112]. This suggests that novel GABAergic synapses form, which could occur as a result of the aberrant sprouting of GABAergic axons[106]. Extrasynaptic $GABA_AR$ 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 has observed decreases in δ subunit expression in these cells[116]. Analyses of hippocampal tissue from patients with TLE reveal alterations in $GABA_AR$ subunit expression patterns that are similar to those observed experimentally[117,118].

Human genetic studies have provided further evidence that abnormal $GABA_AR$ function contributes to epilepsy disorders. Multiple distinct mutations in the γ2[119–121], α1[122,123] and δ subunits[124] have been identified in patients with epilepsy. While the exact mechanisms by which each of these mutations contribute to seizure disorders remain 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 that occurs in the α1 subunit (A322D) leads to subunit retention in the ER, followed by ubiquitin-dependent degradation[128], resulting in overall lower levels of α1-containing $GABA_ARs$ at the cell surface.

Drug abuse

Considerable evidence exists to support a role for $GABA_ARs$ in mediating the addictive properties of drugs of abuse[105,129]. In particular, chronic use of alcohol or benzodiazepines, which are both allosteric modulators of $GABA_ARs$, can lead to drug tolerance, dependence and withdrawal symptoms following drug cessation. Changes in the mRNA and protein expression of various $GABA_AR$ 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 $GABA_AR$ 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 withdrawal[132,133]. This long-term plasticity in $GABA_ARs$ is likely to involve changes in the phosphorylation of $GABA_AR$ subunits and alterations in the endocytosis of specific $GABA_AR$ subtypes. For example, the association of PKC with $GABA_AR$ 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 $GABA_ARs$ contributes to changes in $GABA_AR$ 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 may be responsible for tolerance to both of these drugs. Thus, understanding tolerance-inducing alterations in $GABA_AR$ trafficking should not only advance our understanding of the disease process that leads to alcoholism, but also improve the development of drugs to treat insomnia and anxiety disorders without causing tolerance.

Schizophrenia

Altered expression of several proteins involved in GABAergic transmission have been reported in studies of postmortem tissue from subjects with schizophrenia. Significant reductions in the mRNA levels of GAD-67 (one of the major GABA synthesizing enzymes) and the GABA membrane transporter (GAT-1) have been observed in a subpopulation of interneurons in the prefrontal cortex (PFC) of schizophrenic subjects[137,138]. In addition, a compensatory upregulation of α2-containing $GABA_ARs$ in the axon initial segment of pyramidal neurons has been demonstrated[137]. Reduced GABAergic signaling between these affected interneurons and pyramidal cells has been postulated to contribute to cognitive deficits associated with schizophrenia[104].

The *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 $GABA_AR$ subunit showed select deficits in prepulse inhibition (PPI), which could be normalized by treatment with the antipsychotic haloperidol[139]. Deficits in PPI have been associated with a number of psychiatric disorders, including schizophrenia, and are a measure of a diminished ability for sensorimotor information processing[140]. There was a dramatic loss of synaptic $GABA_ARs$ 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, resulting in an absence of functional inhibitory 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 $GABA_ARs$[143]. Together, these findings suggest that sensorimotor gating is highly sensitive to an imbalance in inhibitory neurotransmission, and that hypofunction of select $GABA_AR$ populations can lead to a schizophrenia-related cognitive impairment. Pharmacological interventions to increase $GABA_AR$ function and/or trafficking of relevant $GABA_AR$ subpopulations may help to alleviate some of the symptoms of schizophrenia and other psychiatric disorders.

7. Conclusions and outlook

Fast inhibitory GABAergic synaptic transmission is a principal determinant of neuronal excitability. This process is dependent upon the delivery of individual $GABA_AR$ 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 $GABA_ARs$ in the ER is an important control point in determining receptor diversity on the plasma membrane. Results from knock-out mice have illustrated preferential receptor subunit partnerships, but how this process is orchestrated remains to be determined. It is becoming apparent that subunits within the ER are subject to activity-dependent ubiquitination, 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 $GABA_AR$ subunits are differentially ubiquitinated, as this may allow neuronal activity to shape the number and pharmacological properties of $GABA_ARs$ on target cells. Modulating receptor palmitoylation, or their binding to accessory proteins as they passage through the Golgi apparatus, may further refine our understanding of how these processes shape the diversity of $GABA_ARs$ on the plasma membrane.

$GABA_ARs$ exhibit high rates of diffusion at the cell surface, which facilitates their delivery to synaptic sites, or entry into coated pits for removal via clathrin-dependent endocytosis. It is emerging that endocytosis is regulated via phosphorylation-dependent mechanisms; more specifically, receptor binding to clathrin-associated proteins can be negatively modulated via the phosphorylation of serine or tyrosine residues within specific $GABA_AR$ subunits. This could allow cell-signaling pathways that regulate $GABA_AR$ phosphorylation to also influence their cell surface stability. The relevance of these processes awaits the development of knock-in mouse lines in which the phosphorylation residues within individual AP2 binding motifs have been ablated. However, it is interesting to note that dephosphorylation of $GABA_ARs$ and their enhanced endocytosis may be responsible for compromised synaptic inhibition during *status epilepticus*. 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 $GABA_ARs$ remains rudimentary.

The 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 containing α2 and γ2 subunits. There is also evidence that α1-containing $GABA_A$Rs, although tightly co-localized 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 are capable of binding specific gephyrin splice variants, and the roles that these binding motifs play 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 central nervous system pathologies, ranging from epilepsy to schizophrenia. This information is also likely to lead to the identification of novel therapeutic drug targets that will allow for the pharmacological modulation of individual $GABA_A$R subtypes.

Biographies

Tija C. Jacob

Tija C. Jacob obtained her Ph.D. degree from the University of California at Berkeley in the lab of Joshua M. Kaplan, studying neurotransmitter and neuropeptide modulation of behavior in the nematode *C. elegans*. She was a postdoctoral fellow with Steve J. Moss at University College London, UK, and is currently continuing her training in his lab at the University of Pennsylvania, Philadelphia, USA. Her research interests focus on regulation of $GABA_A$ receptor intracellular trafficking, cell surface mobility, and interactions with the inhibitory scaffold and how these processes are altered by disease or drug treatment.

Stephen J. Moss

Stephen J. Moss obtained his PhD in the laboratory of Eric Barnard at Imperial College London and the MRC-laboratory of Molecular Neurobiology in Cambridge. After postdoctoral training with British Biotechnology in Oxford, Richard Huganir at Johns Hopkins University and Martin Raff at University College London (UCL) he was appointed as a group leader in the MRC laboratory of Molecular Biology at UCL in 1994. In 2000 he was promoted to Professor of Molecular Pharmacology, in the Pharmacology Department at UCL. He joined the University of Pennsylvania as a Professor of Neuroscience in 2003. His research interests centre on the control of synaptic inhibition with particular emphasis on the functional modulation of metabotropic and ionotropic GABA receptors.

Rachel Jurd

Rachel Jurd obtained her Ph.D. at the Institute of Pharmacology and Toxicology, University of Zurich, Switzerland, in the laboratory of Dr. Uwe Rudolph, studying $GABA_A$ receptors and general anaesthetic mechanisms. She then pursued postdoctoral training at the Ernest Gallo Clinic and Research Center, University of California, San Francisco, USA, before joining Dr. Stephen J. Moss's laboratory at the University of Pennsylvania, Philadelphia, USA, in 2007. Her research focuses on the regulation of $GABA_A$ receptor trafficking by phosphorylation, as well as on the molecular mechanisms underlying drug tolerance.

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Glossary

BENZODIAZEPINES, Pharmacologically active molecules with sedative, anxiolytic, amnesic and anticonvulsant effects. They act by binding at the interface between α(1,2,3 or 5) and γ subunits of $GABA_A$ receptors to potentiate the response elicited by GABA.; YEAST TWO-HYBRID SCREEN, System used to determine the existence of direct interactions between two proteins. It involves the expression of two proteins in yeast; the plasmids encoding these proteins are fused to GAL4 DNA-binding and GAL4 activation domains, respectively. If the proteins interact, the resulting complex will drive the expression of a reporter gene, commonly β-galactosidase.; GABAergic PLASTICITY, Changes in local activity that lead to longer-term increases or decreases in inhibitory synaptic strength.; UBIQUITIN-PROTEASOME SYSTEM, Ubiquitin is a 76 amino-acid protein that serves as a tag to mark proteins destined for degradation. Proteins tagged by a polyubiquitin chain are targeted to the proteasome, a large, multimeric barrel-like complex that acts by proteolysis to degrade proteins.; PALMITOYLATION, The covalent attachment of a palmitate (16-carbon saturated fatty acid) to a cysteine residue through a thioester bond.; CLATHRIN, One of the main protein components of the coats formed during membrane endocytosis.; AP2 COMPLEX, Tetrameric complex composed of subunits called adaptins that play an important role in clathrin-dependent membrane endocytosis.; RNA interference (RNAi), A molecular method in which small interfering RNA sequences are introduced into cells or tissues, and subsequently decrease the expression of target genes.; 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.; TONIC INHIBITION, An inhibitory response that is mediated by the activation of extra- or peri-synaptic $GABA_A$ receptors through ambient concentrations of GABA..

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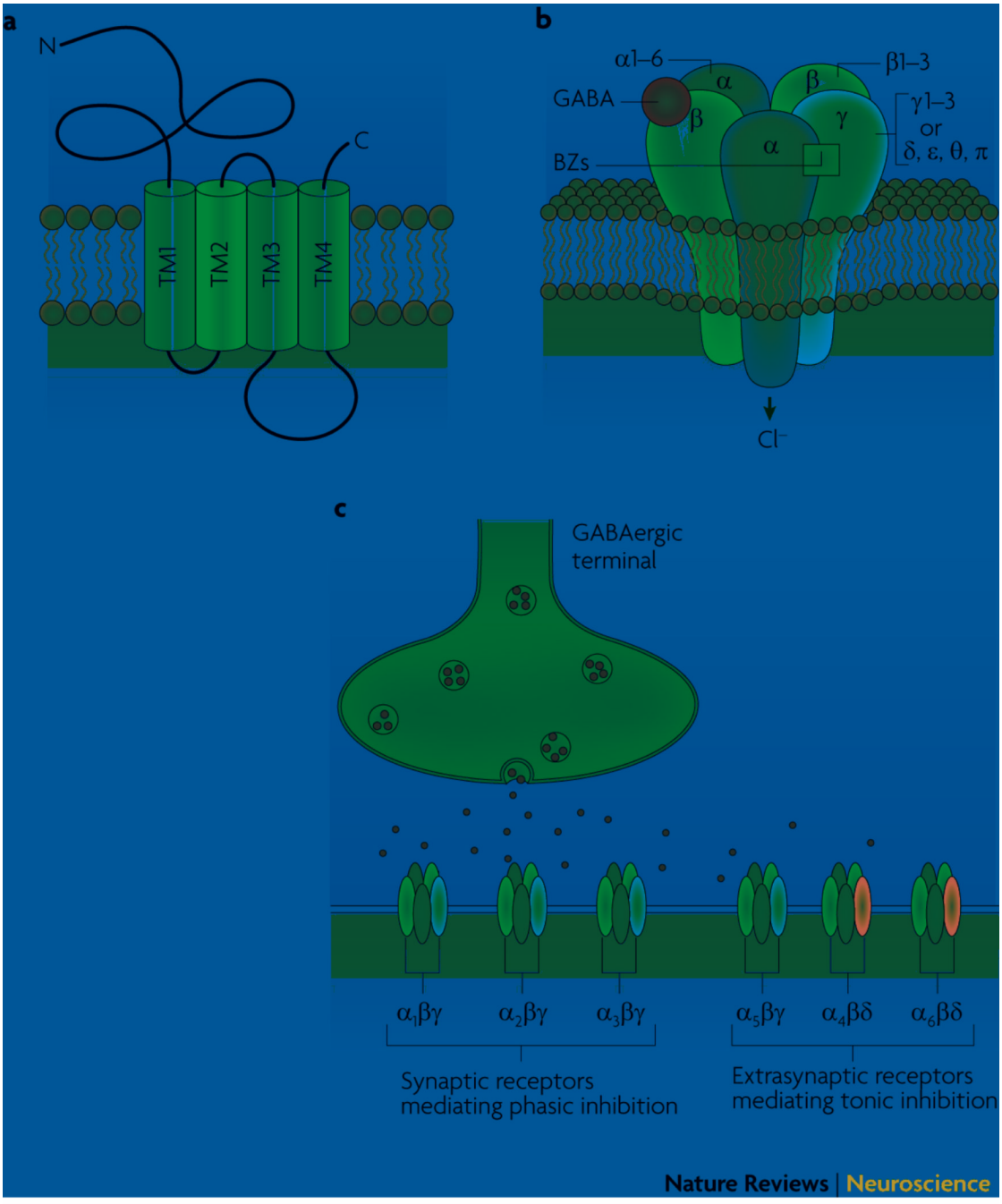

Figure 1. $GABA_A$ receptor structure and neuronal localization

(A) $GABA_A$ receptors are members of the ligand-gated ion channel superfamily. Receptor subunits consist of four hydrophobic transmembrane (TM1–4) domains, where TM2 is believed to line the pore of the channel. The large extracellular N-terminus is the site for the binding of the neurotransmitter GABA, as well as containing binding sites for psychoactive drugs, such as benzodiazepines (BZ). Each receptor subunit also contains a large intracellular domain between TM3 and TM4, which is the site for various protein interactions as well as the site for various post-translational modifications that modulate receptor activity. **(B)** Five subunits from 7 subunit subfamilies (α,β,γ,δ,ε,θ,π) assemble to form a heteropentameric chloride-permeable channel. Despite the extensive heterogeneity of $GABA_A$ receptor subunits,

the majority of $GABA_A$ receptors expressed in the brain consist of 2α, 2β, and 1γ subunit, where the γ subunit can be replaced by δ, ε 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 chloride ions. BZ-binding occurs at the interface between α(1,2,3 or 5) and γ subunits and potentiates GABA-induced chloride flux. **(C)** $GABA_A$ receptors 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. Receptors composed of the aforementioned subunits are benzodiazepine-sensitive. In contrast, receptors composed of α(4,6)βδ are benzodiazepine-insensitive, and are localized at extrasynaptic sites.

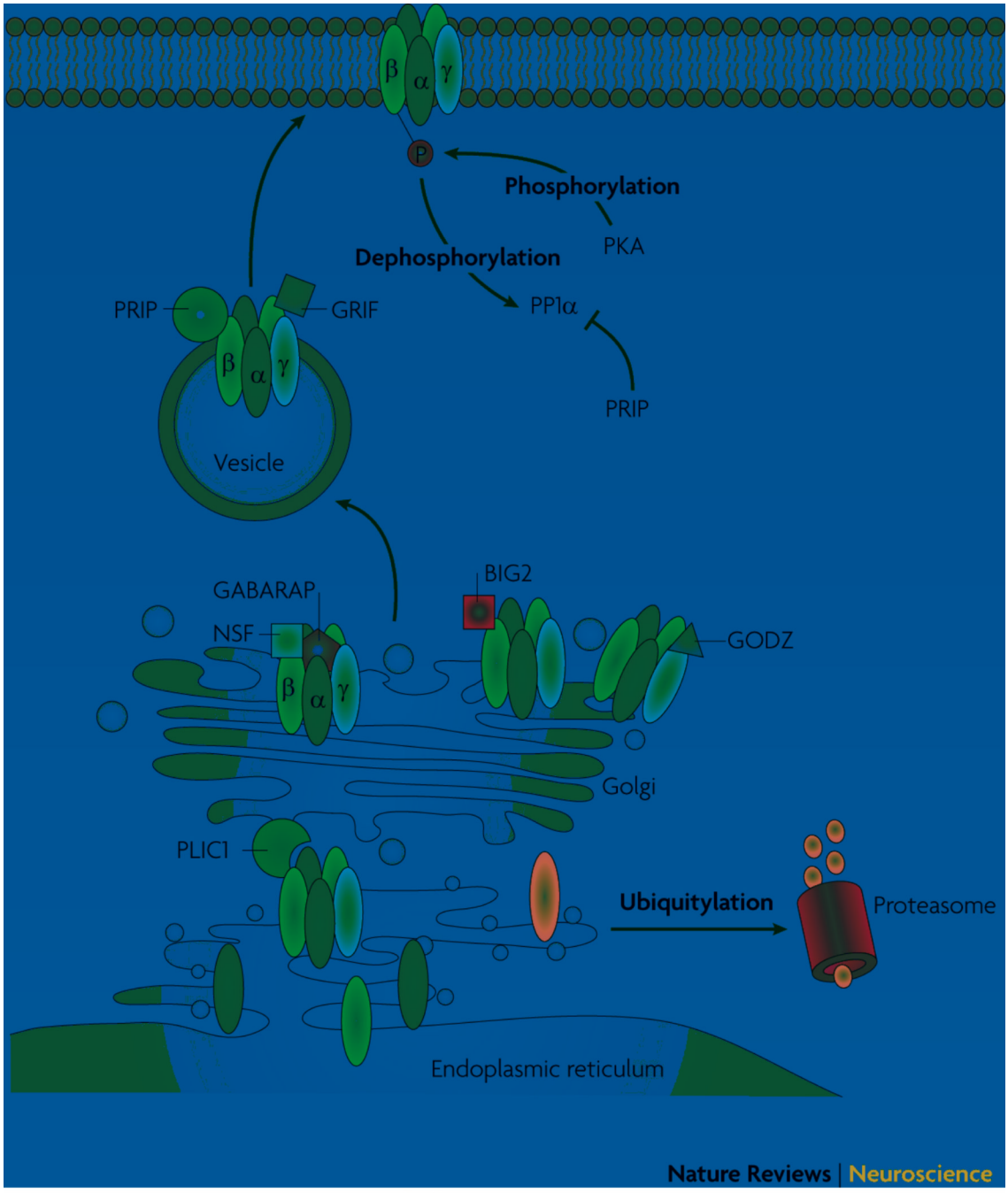

Figure 2. Trafficking of $GABA_A$ receptors

$GABA_A$ receptor subunits are synthesized and assembled into pentameric structures in the endoplasmic reticulum (ER). This process is carefully regulated in the ER. The fate of $GABA_A$ receptor subunits can be modulated by ubiquitination and subsequent ER-associated degradation via the proteasome. Ubiquitinated $GABA_A$ receptor subunits can also be modulated via their association with Plic-1. Plic-1 facilitates $GABA_A$ receptor accumulation at the synapse by preventing the degradation of ubiquitinated $GABA_A$ receptors. Exit into the Golgi network and subsequent trafficking to the plasma membrane are also facilitiated by a number of $GABA_A$ receptor-associated proteins. GABARAP associates with the γ2 subunit of $GABA_A$ receptors and aids in the trafficking of receptors from the Golgi network to the plasma

membrane. NSF and BIG2 are also localized to the Golgi network, where they bind to β subunits of $GABA_A$ receptors and modulate receptor trafficking. Palmitoylation of γ subunits occurs in the Golgi apparatus as a result of an association with the palmitoyltransferase, GODZ, and is a critical step in the delivery of $GABA_A$ receptors to the plasma membrane. GRIF proteins play a role in the trafficking of $GABA_A$ receptors to the membrane. PRIP proteins also play essential roles in the trafficking of $GABA_A$ receptors, as well as in modulating the phosphorylation state of $GABA_A$ receptors.

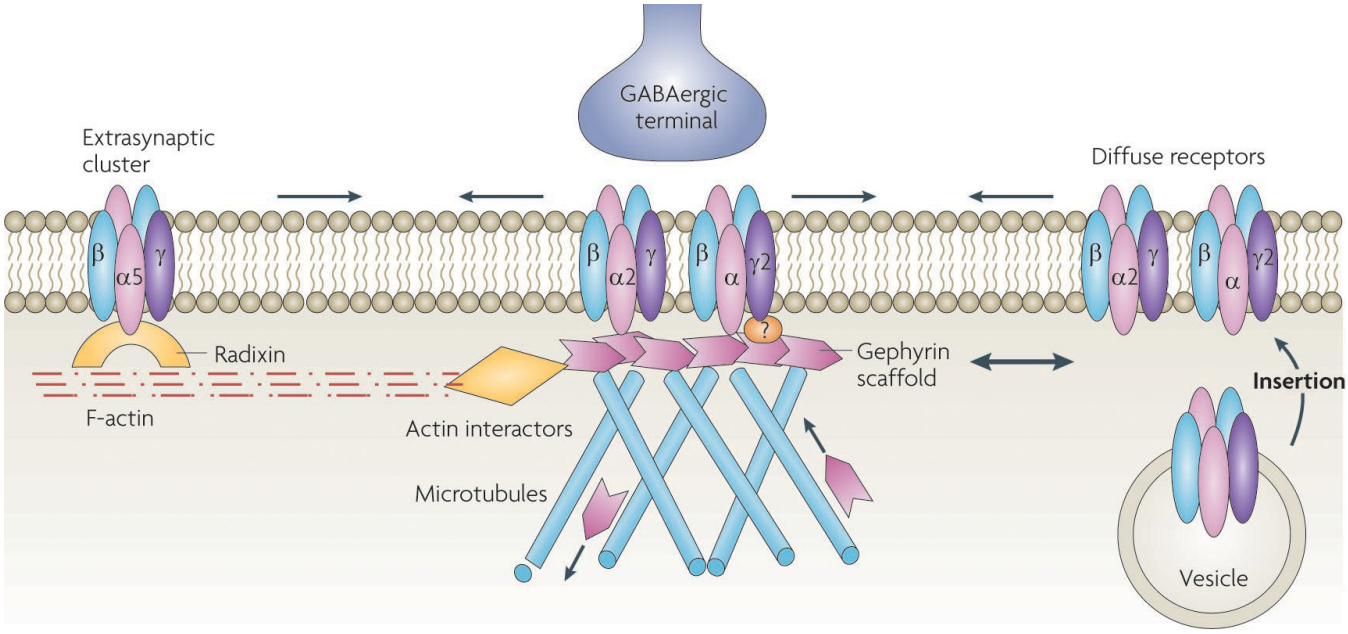

Figure 3. Dynamic regulation of receptor lateral mobility at the GABAergic synapse

$GABA_A$ receptors are inserted into the plasma membrane at extrasynaptic sites, where they can then diffuse into synaptic sites. Lateral diffusion (black arrows) within the plasma membrane allows for 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 $GABA_A$ receptors 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)[144] and profilins 1 and 2[144,145]. No direct interaction between gephyrin and the γ2 subunit has been demonstrated. However, gephyrin depletion increases γ2 cluster mobility, and loss of the γ2 subunit results in post-synaptic sites devoid of gephyrin. This suggests an unidentified intermediary interactor or a post-translational modification that could link γ2-containing receptors and gephyrin. Alternatively, clustering of γ2-containing receptors might occur via an independent mechanism. Gephyrin also displays local lateral movements (red double arrow), and removal or addition by microtubule dependent trafficking, contributing additional mechanisms to regulate synaptic transmission. Extrasynaptic localization of α5-containing $GABA_A$ receptors is controlled by binding to activated radixin, which directly binds F-actin.

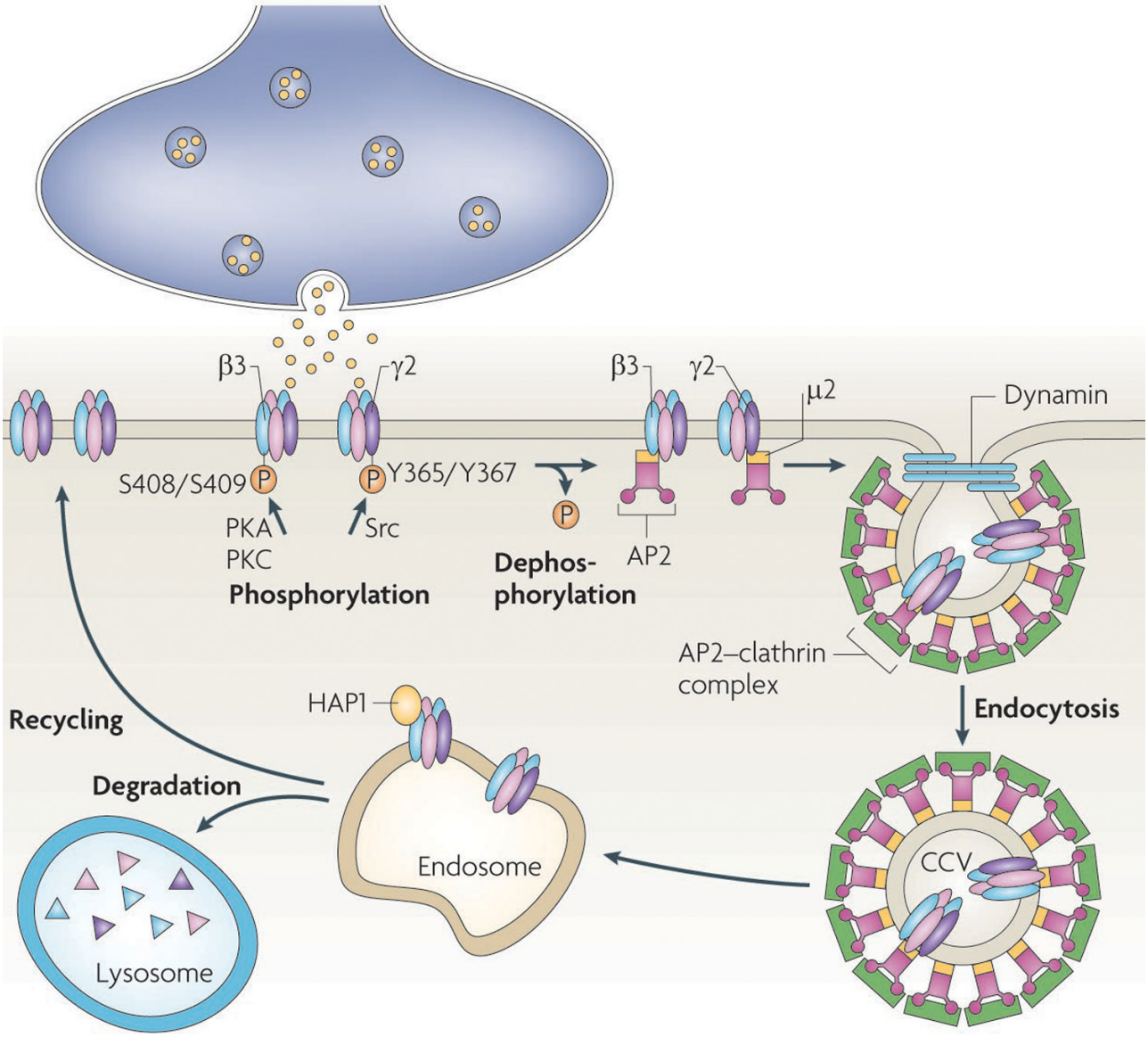

Figure 4. Regulation of $GABA_A$ receptor endocytosis and post-endocytic sorting

Clathrin-dependent endocytosis is the major internalization mechanism for neuronal $GABA_A$ receptors. The intracellular loops of β and γ subunits interact with the clathrin adaptor protein 2 (AP2) complex. Binding of the μ2 subunit is inhibited by phosphorylation of the AP2-interacting motifs in $GABA_A$ receptor subunits, increasing cell surface receptor levels and enhancing the efficacy of inhibitory synaptic transmission. Once endocytosed in clathrin-coated vesicles (CCV), the vesicles are uncoated and fuse with early or sorting endosomes, resulting in $GABA_A$ receptors being subsequently recycled to the plasma membrane or degraded in lysosomes. Huntingtin associated protein-1 (HAP1) interacts with β subunits and promotes receptor recycling to the plasma membrane. PKA and PKC regulate phosphorylation of serine residues 408/9 in the AP2-binding motif of β3 subunits, while tyrosine residues 365/7 in the γ2 subunit are phosphorylated by Src kinase.

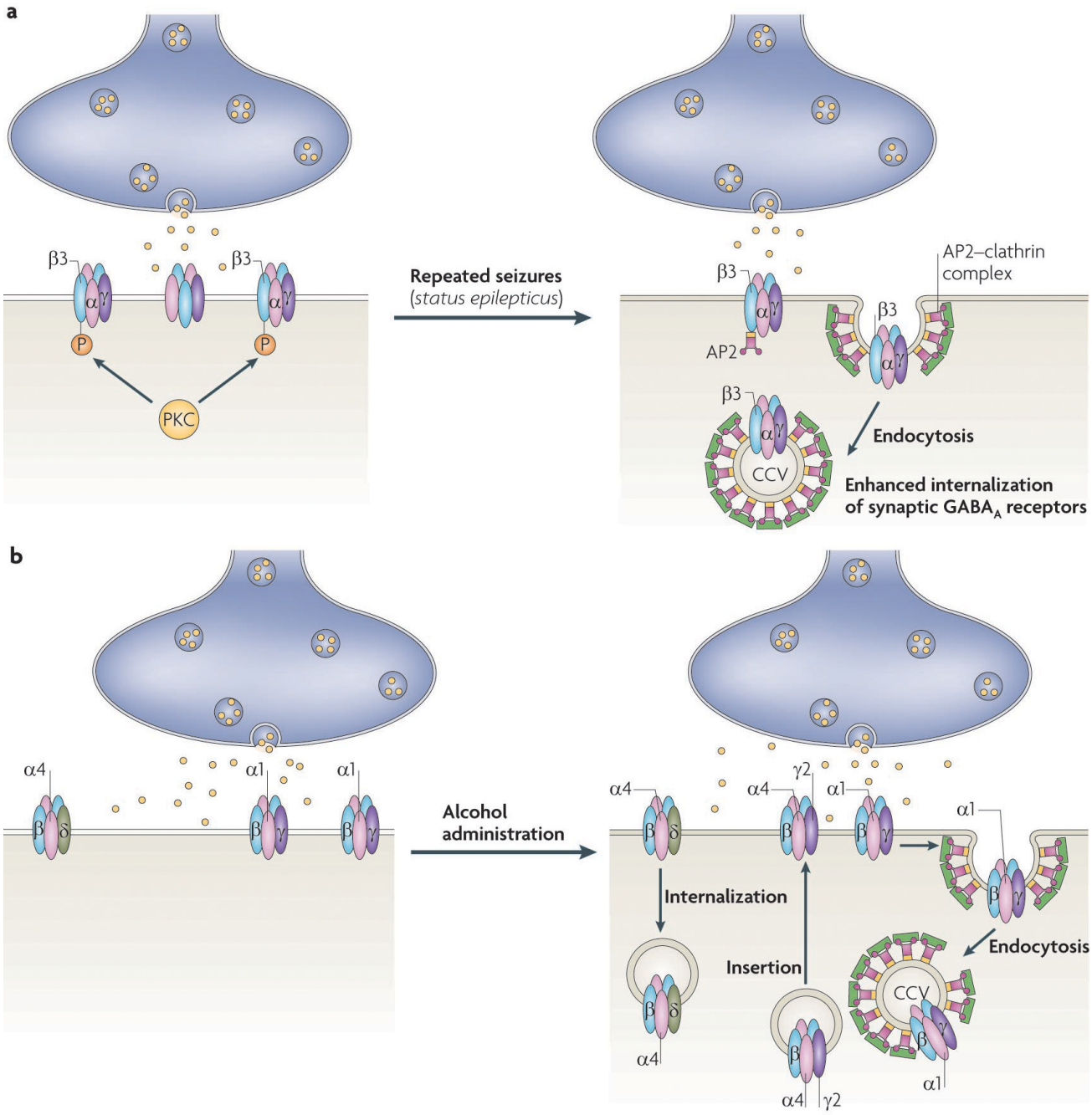

Figure 5. Dysregulation in GABA$_A$ receptor trafficking in neurological disease

(A) Repetitive, non-abating seizures that lead to *status epilepticus* result in a decrease in the phosphorylation of GABA$_A$ receptor β subunits by PKC. This leads to an increased associated with the clathrin adaptor AP2, followed by increased internalization via clathrin-mediated endocytosis. Decreased numbers of synaptic GABA$_A$ receptors lead to reduced synaptic inhibition (ie. increased excitatory drive and a lower seizure threshold) as well as decreased benzodiazepine sensitivity. **(B)** Alcohol-induced plasticity in GABA$_A$ receptors involves changes in both synaptic and extrasynaptic GABA$_A$ receptor populations. After alcohol administration, there is an increased internalization of δ-containing extrasynaptic GABA$_A$ receptors. There is also an increased internalization of α1-containing synaptic GABA$_A$

receptors via clathrin-dependent internalization. Insertion of distinct $GABA_A$ receptor populations at synaptic sites (ie. $\alpha4\beta\gamma2$) have been hypothesized to serve a compensatory role at inhibitory synapses, however, these receptors differ in their physiological functions from normal synaptic $GABA_A$ receptor populations, as well as being benzodiazepine-insensitive.