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Regulating the Efficacy of Inhibition Through Trafficking of γ -Aminobutyric Acid Type A Receptors

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Abstract

Trafficking of anesthetic-sensitive receptors within the plasma membrane, or from one cellular component to another, occurs continuously. Changes in receptor trafficking have implications in altering anesthetic sensitivity. γ -aminobutyric acid type A receptors (GABA_ARs) are anion permeable ion channels and are the major class of receptor in the adult mammalian central nervous system that mediates inhibition. GABAergic signaling allows for precise synchronized firing of action potentials within brain circuits that is critical for cognition, behavior, and consciousness. This precision depends upon tightly controlled trafficking of GABA_ARs into the membrane. General anesthetics bind to and allosterically enhance GABA_ARs by prolonging the open state of the receptor and thereby altering neuronal and brain circuit activity. Subunit composition and GABA_AR localization strongly influence anesthetic endpoints, therefore, changes in GABA_AR trafficking could have significant consequences to anesthetic sensitivity. GABA_ARs are not static membrane structures but are in a constant state of flux between extrasynaptic and synaptic locations and are continually endocytosed and recycled from and to the membrane. Neuronal activity, post-translational modifications, and some naturally occurring and synthetic compounds can influence the expression, and trafficking of GABA_ARs. In this article we review GABA_ARs, their trafficking and how phosphorylation of GABA_AR subunits can influence the surface

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expression and function of the receptor. Ultimately, alterations of GABA_AR trafficking could modify anesthetic endpoints, both unintentionally through pathological processes but potentially as a therapeutic target to adjust anesthetic-sensitive GABA_ARs.

Introduction

The information regarding synaptic γ -aminobutyric acid type A receptor (GABA_ARs) (GABA_AR) trafficking is limited compared to that known for glutamatergic receptors. Trafficking of extrasynaptic GABA_ARs is even less well described. This lack of understanding of the control and transport of this type of GABA_AR is important considering that extrasynaptic GABA_ARs appear to be a particularly key target for anesthetic agents (1–5). Increasing the trafficking of α 4-containing GABA_ARs to extrasynaptic sites leads to an increase in tonic inhibitory current, enhancing the hypnotic and amnestic actions of etomidate and propofol (6). Exposure to etomidate and isoflurane has been recently described to have prolonged effects on cell surface expression of α 5-containing GABA_ARs with a resulting increase in anesthetic sensitivity and memory impairment (7). Similarly, the proinflammatory cytokine, interleukin-1 β increases the trafficking of α 5-containing GABA_ARs to the surface of hippocampal neurons increasing the sensitivity to the immobilizing and memory impairment properties of general anesthetics (8,9). Surface levels of extrasynaptic GABA_ARs are altered in many disease states such as neurodevelopment disorders, alcoholism, and in brain injury (10–12). A better understanding of the processes underlying the mechanism of GABA_ARs trafficking could pave the way for novel therapeutic targets that reverse pathological levels of receptor expression (13).

This review will briefly summarize GABA_AR structure and formation followed by descriptions of receptor trafficking and how phosphorylation can alter membrane expression of GABA_AR subunits. For a more in-depth review of synaptic GABA_AR trafficking and phosphorylation see Luscher et al. (14) and Nakamura et al. (15). In addition, we have attempted to emphasize the dynamics of GABA_AR trafficking, the speed of which is often overlooked but has the potential to dramatically alter GABA_AR number and subunit composition. Targeting receptor insertion and endocytosis could have fast and prolonged effects on GABA_AR pharmacology.

GABA mediates the majority of CNS inhibitory neurotransmission

In the adult mammalian central nervous system (CNS), γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter. It is estimated that approximately one-quarter of all neurons use GABA as their primary neurotransmitter (16). GABA is synthesized in the brain from the decarboxylation of the excitatory neurotransmitter glutamate in a reaction catalyzed by L-glutamic acid decarboxylase (GAD) (17). In the mammalian brain, GAD is encoded by two different genes to produce two isoforms with distinct expression patterns. GAD65 is restricted to nerve terminals where it is involved with GABA production for neurotransmission whereas GAD67 expression is observed throughout the cell and is involved in GABA production for non-neurotransmission functions such as synaptogenesis and neuroprotection (18). Following synthesis, GABA is transported into synaptic vesicles

by the vesicular inhibitory amino acid transporter (VIATT) where it becomes available for release into the synaptic cleft. The actions of GABA are terminated by reuptake via the GABA transporters (GAT-1 – GAT-4), also known as sodium- and chloride-dependent GABA transporter 1–4 (19). In humans, the majority of GABA reuptake is mediated by GAT-1 expressed at nerve terminals and astrocytic processes in neurons and glia, respectively (20,21).

GABA_A receptors mediate fast inhibition in the CNS

GABA_ARs are ligand-gated chloride channels responsible for mediating fast inhibition in the CNS and are the prominent neurotransmitter receptor to which most general anesthetics bind and modulate. GABA binding generates a conformational change in the protein's intrinsic chloride channel, allowing chloride ions to flow down its electrochemical gradient. During early development, high intracellular chloride concentrations result in a depolarizing response to GABA (22–24). In adult brains, the efflux of chloride by the K⁺/Cl[−] cotransporter, KCC2, leads to lower intracellular chloride concentrations resulting in GABA producing a hyperpolarizing response which reduces the probability that an action potential will be generated.

GABA_ARs belong to the cys-loop ion channel superfamily whose other members include the nicotinic acetylcholine receptors, glycine receptors, 5-HT₃ receptors and zinc-activated channels (25). Cys-loop receptors are heteropentamers with an intrinsic ion channel assembled from various classes of subunits; 19 GABA_AR subunits have been identified that belong to 8 different classes: α(1–6), β(1–3), γ(1–3), δ, ε, π, θ, and ρ(1–3)2 (26,27). The GABA binding site is located at the interface between α and β subunits with two agonist binding sites per receptor while the benzodiazepine binding site lies at the interface between α and γ subunits (25).

GABA_AR subunits possess similar structures: a large extracellular N-terminal containing the characteristic cysteine-cysteine loop, four transmembrane domains (TM1-TM4), a large intracellular loop between TM3 and TM4 that is the most genetically divergent part of each subunit and a short extracellular C-terminal (25,28). The intracellular loop dynamically regulates receptor activity by altering its trafficking and cell surface expression through protein-protein interactions with regulatory molecules such as microtubule-binding proteins, kinase-anchoring protein and cytoskeletal proteins and acts as a site for post-translational modifications including ubiquitination and phosphorylation (15,29).

GABA_AR containing specific subunits display distinct cellular and subcellular expression

GABA_AR subunit composition influences GABA_AR trafficking and therefore, its cellular and subcellular localization. In addition, subunit composition establishes the physiological and pharmacological properties of the receptor (26,30). Despite the potential for a wide variety of possible subunit combinations in the assembly of GABA_ARs, *in vivo* expression of functional GABA_AR subunit combinations is limited, being regulated both spatially and temporally (26). Within the CNS, GABA_AR subtypes display distinct cellular and

subcellular localization patterns (31–33). The most commonly encountered GABA_AR is composed with a stoichiometry of 2 α , 2 β and one γ subunit (27,31,32,34). At sites further away from inhibitory synapses, or extrasynaptic sites, the δ subunit can replace γ to form GABA_ARs with pharmacological properties that are distinct from γ -containing GABA_ARs (12,35,36).

GABA_AR trafficking

To maintain the efficacy of inhibition GABA_AR subunits need to be packaged together to form pentameric receptors, trafficked to the cell surface and then move to the either synaptic or extrasynaptic locations. The low ambient GABA in extracellular spaces is not sufficient to activate synaptic type GABA_ARs, which are required to be localized at synaptic locations. Similarly, cells need to have extrasynaptic type receptors in the correct place in order for them to generate tonic inhibition current.

GABA_AR oligomerization and transport

After translation, GABA_ARs are assembled within the endoplasmic reticulum (ER). Although the heteropentamer can be formed from many combinations of the 19 available subunits, *in vivo* studies have established that the number of combinations that can be properly assembled and trafficked to the membrane is reasonably limited (28,37). For example, although GABA_ARs composed of only α and β subunits are functional in expression studies and may occur *in vivo* where they are proposed to mediate tonic inhibition, $\alpha\beta\gamma$ -GABA_AR and $\alpha\beta\delta$ -GABA_AR combinations are the most commonly observed GABA_ARs *in vivo* (38–42).

In the ER, resident chaperone proteins such as calnexin and binding immunoglobulin protein (BiP) facilitate the proper folding and assembly of the receptor; improperly assembled or misfolded receptors are directed towards degradation in proteasomes (43–46). The negative regulator of proteasomal degradation, Plic-1 (protein that links integrin associated protein with the cytoskeleton-1) assists in stabilizing the receptor within the ER and facilitates its transport to the Golgi apparatus following assembly (47). Plic-1 interacts with the intracellular domains of GABA_AR α and β subunits to regulate their transport through the secretory pathway (48). In the Golgi, GABA_ARs are further processed and sorted into secretory vesicles for insertion into the plasma membrane. The γ 2-containing GABA_ARs are palmitoylated in the Golgi through serine residues within γ 2 cytoplasmic domain by the Golgi-specific DHHC zinc finger protein (GODZ), a process critical for its accumulation at inhibitory synapses (49).

Trafficking of GABA_ARs from the Golgi through secretory vesicles to the plasma membrane is promoted by interaction with cytoplasmic proteins such as BIG2 (brefeldin A-inhibited guanine nucleotide exchange factor 2), GABARAP (GABA_AR-associated protein), phospholipase C-related catalytically inactive proteins 1 and 2 (PRIP1/2) and N-ethylmaleimide sensitive factor (NSF). BIG2 assists in the translocation of proteins through the trans-Golgi network through interactions with the intracellular domains of GABA_AR β subunits although it has also been localized to endosomes and may influence endocytic

recycling of GABA_ARs (50,51). GABARAP, a member of the membrane-associated proteins (MAPs) interacts with microtubules and the intracellular domains of GABA_AR γ subunits (52,53). Overexpression of GABARAP in expression systems and cultured neurons increases the membrane expression of γ 2-containing GABA_ARs, suggesting that GABARAP may facilitate the translocation of GABA_ARs from intracellular compartments to the cell membrane (52,54,55). The evidence for this is further supported by the finding that phospholipase C (PLC)-related catalytically inactive protein PRIP1 and PRIP2, competitively inhibits γ 2 binding to GABARAP (56). As its name suggests, PRIPs are proteins related to phospholipase C but lack their catalytic activity. PRIP1 and PRIP2 double knockout mice exhibit reduced diazepam sensitivity and decreased surface benzodiazepine binding sites, indicative of a reduction in the membrane surface expression of γ 2-containing GABA_ARs (56–58). PRIP1 may also indirectly influence membrane trafficking of GABA_ARs by binding to and inhibiting protein phosphatase 1a (PP1a) (59). In addition, PRIP1 binds to the intracellular domains of the GABA_AR β subunits (59). The hexameric ATPase, NSF also directly binds the intracellular domains of GABA_AR β subunits to increase receptor trafficking towards the membrane (60). In addition, NSF interacts with GABARAP within the Golgi apparatus where it is proposed to facilitate the intracellular trafficking of GABA_ARs (61).

Targeting of GABA_ARs to inhibitory synapses

Through controlling the availability and numbers of GABA_ARs at the plasma membrane, GABA_AR trafficking between intracellular compartments and the membrane is the determining factor in regulating GABAergic inhibition. At extrasynaptic sites, GABA_ARs are inserted into the plasma membrane following *de novo* assembly in the ER or reinserted after internalization (62,63). Following insertion into extrasynaptic sites, the lateral diffusion of GABA_ARs in the lipid bilayer under Brownian diffusion is rapid; single-particle tracking experiments have measured the diffusion coefficient at extrasynaptic sites to be $0.2 \mu\text{m}^2\text{s}^{-1}$. This is in contrast to the diffusion coefficient at postsynaptic membrane, $0.03 \mu\text{m}^2\text{s}^{-1}$ (64). The lateral diffusion of GABA_ARs to synaptic or extrasynaptic sites is influenced predominantly by subunit composition along with protein-protein interactions with scaffolding proteins such as gephyrin and cytoskeletal proteins such as radixin (28,45).

Gephyrin, the key scaffolding protein at inhibitory synapses for glycine and GABA_ARs, is involved in the clustering of synaptic GABA_AR subtypes at GABAergic synapses (65). Gephyrin directly binds to the intracellular domains of α 1, α 2, α 3, α 5, β 2, β 3 and γ 2 subunits to anchor synaptic GABA_AR subtypes to the cytoskeleton (27,34,66). Experiments with gephyrin knockout mice, along with antisense oligonucleotide knockdown of gephyrin, have demonstrated that inhibiting gephyrin expression significantly decreases γ 2 punctate staining and GABA_AR clustering at inhibitory synapses (67–70). Likewise, gephyrin clustering along with synaptic GABA_AR clustering were abolished in γ 2 knockout mice, suggesting an interdependence between gephyrin and synaptic GABA_ARs in the formation of inhibitory synapses (67). In addition to gephyrin, interactions with synaptic adhesion molecules such as collybistin, neuroligin and neuroligin promote the formation of inhibitory synapses. Collybistin, a membrane-associated guanine-nucleotide exchange factor (GEF), directly binds gephyrin to facilitate its translocation to the plasma membrane surface

(71,72). Presynaptically localized β -neurexin and postsynaptically localized neuroligin 2 (NL2) promote inhibitory synaptogenesis by facilitating the proper alignment of the inhibitory synapse (27,37).

Less is known about the mechanisms for anchoring extrasynaptic GABA_ARs to the cytoskeleton. The adaptor protein radixin, a member of the ezrin/radixin/moesin family, directly links the extrasynaptic $\alpha 5$ subunit to the actin cytoskeleton although its functional relevance has not been determined since depleting radixin in neurons abolished $\alpha 5$ subunit clustering but had no effect on $\alpha 5$ surface expression or GABA-mediated tonic currents. However, radixin depletion, or reducing the $\alpha 5$ subunit clustering through a radixin point mutant that prevents activation via phosphorylation did alter synaptic miniature inhibitory postsynaptic currents (mIPSCs). The mIPSCs were more frequent and slower decaying suggesting that the $\alpha 5$ subunit was now contributing to synaptic GABA_ARs (73,74). The radixin-depleted mice also had memory impairments that occurred due to the prolonged synaptic inhibitory current (74). These studies demonstrate that pools of extrasynaptic $\alpha 5$ subunit-containing GABA_ARs can be trafficked to synaptic sites to directly change cellular and behavior properties.

GABA_AR endocytosis, recycling, and degradation

Internalization of GABA_ARs occurs at extrasynaptic sites by binding of the clathrin adaptor protein AP2 to the intracellular domain of the receptor. AP2 is a heterotetrameric protein composed of four subunits commonly called adaptins: α , $\beta 2$, $\mu 2$ and $\sigma 2$. The α and $\beta 2$ adaptins bind to the plasma membrane and clathrin and are involved in the recruitment of accessory proteins to facilitate the assembly of clathrin-coated pits, $\mu 2$ adaptin is responsible for cargo recognition and the $\sigma 2$ adaptin stabilizes the adaptor complex (75–78).

The $\mu 2$ adaptins responsible for cargo binding recognize at least four motifs within GABA_ARs: a dileucine motif in the GABA_AR $\beta 2$ subunit (L³⁴³L³⁴⁴), a 10 amino acid atypical AP2 binding motif enriched with lysine and arginine residues within the intracellular domain in all β subunits, and in $\gamma 2$ subunits: a 12 amino acid domain analogous to the β subunit AP2 binding site and a tyrosine based YGYECL motif (44,79–83). The AP2 binding sites within the β and $\gamma 2$ subunits overlap with the major phosphorylation sites for PKA and PKC along with calcium/calmodulin-dependant kinase II (CAMKII) and AKT for the β subunits and Fyn and Src-family of tyrosine kinase for the $\gamma 2$ subunit: S409 for $\beta 1$, S410 for $\beta 2$, S408/9 for $\beta 3$ and Y365/367 for $\gamma 2$ (84–93). Following endocytosis, GABA_ARs can undergo ubiquitination and degradation within lysosomes or recycled for reinsertion back to the plasma membrane. Interactions with the cytoplasmic domains of β and γ subunits with lysosomal regulatory proteins such as Huntingtin-associated protein 1 (HAP1) and calcium modulating cyclophilin ligand (CAML) promote the recycling of receptors to the membrane surface (28,54,94,95).

Dynamics of GABA_AR trafficking

In an unperturbed system, the steady-state responses observed during the course of an experiment give the impression of a stable receptor number at the cell surface. However,

GABA_AR insertion and endocytosis are constantly ongoing allowing the neuron to finely tune the level of inhibition on a time scale that allows rapid adaptation. We describe here some key experimental estimates of the chronology of GABA_AR trafficking. (Figure 1)

Blocking endocytosis with the internally dialyzed peptide P4 (targets the function of the GTPase dynamin, which is responsible for endocytosis) resulted in an increase in hippocampal mIPSC amplitudes due to an increase in membrane GABA_ARs at the synapse. An increase was detected in less than 10 min after the establishment of the whole-cell configuration and reached a plateau at approximately 2 fold higher than control after 40–50 min (44,83).

Using the peptide (pepβ3) which represents the minimal μ2-binding region in the GABA_AR β3 subunit, the endocytosis of β3 subunit containing GABA_ARs are reduced by competing with μ2. This peptide also increased mIPSC amplitudes; the increase being detected 10 min after pepβ3 was dialyzed into the neuron following the establishment of the whole-cell configuration. In hippocampal slices an increase in mIPSC amplitude was noted in CA1 neurons with pepβ3 dialysis within minutes following attainment of the whole-cell configuration (96).

In whole-cell recordings from cortical neurons, the mIPSC amplitude can be steady for over 60 mins. However, including in the pipette a peptide (YECL-pep) which competes with the binding of μ2 to γ2 subunits resulted in an increase in mIPSC that was detected after 10 min (80). When the peptide was chemically phosphorylated it failed to affect mIPSC amplitude demonstrating the role of phosphorylation.

In experiments with cortical neurons maintained in culture, biotinylation experiments using immunoblotted with antibodies against GABA_AR β3 subunit have demonstrated that for a given population of surface GABA_ARs, constitutive endocytosis removes approximately 20–25% of total surface GABA_ARs within 30 min. Of these internalized GABA_ARs ~30% are recycled back to the plasma membrane within 5 min and ~70% are back within an hour (97). Internalized GABA_ARs can undergo lysosomal degradation but this process is much slower with ~30% of the initially labeled GABA_ARs undergoing lysosomal degradation within 6 hours.

Bogdanov et al.⁶² nucleofected cDNA encoding β3 subunits containing the bungarotoxin (Bgt) binding site peptide together with a green fluorescent pHlurin reporter into freshly isolated hippocampal neurons. After 12–14 DIV neurons were exposed to red fluorescent rhodamine-conjugated Bgt (Rd-Bgt) then washed in unlabeled Bgt and the neurons fluorescence along with synaptic labeling was measured. They found that extrasynaptic GABA_ARs have a shorter residence time (a significant decrease within 10 min from the start of the experiment) on the cell surface compared to synaptic GABA_ARs (62). The same group examined insertion of GABA_ARs with Rd-Bgt in neurons whose constitutive endocytosis had been blocked and found newly inserted β3 subunit containing GABA_ARs in the extracellular region within 5 min. This measurement for the insertion is consistent with other data from recombinant cells and neurons (47,98).

There appears to be remarkable agreement in the timing of these finely orchestrated trafficking events. Biochemical, imaging, and electrophysiological experiments are consistent in demonstrating a significant endocytosis occurring within 10 minutes, insertion 5 to 10 min, and the majority of surface GABA_ARs being recycled within an hour.

Alterations of trafficking by phosphorylation

Phosphorylation dynamically regulates the membrane expression of β - and γ 2-containing GABA_ARs by inhibiting AP2 binding leading to an increase in surface β - and γ 2-GABA_AR subtypes (44,99,100). When S408/9 of the GABA_AR β 3 subunit is not phosphorylated, the μ 2 adaptin is able to bind with high affinity; phosphorylation of S408/9 significantly reduced μ 2 binding to β 3 subunit by 100 fold (29,44). As a result of this phosphorylation-mediated stabilization of β 3-containing GABA_ARs there was an increased GABAergic inhibition by increasing mIPSC amplitude and frequency (29). Therefore, phosphorylation of β 3 within the AP2 binding motif reduced μ 2 binding to the β 3 subunit and increased its surface expression and GABAergic phasic inhibition. Similar to the PKC phosphorylation of the β 3 subunit, μ 2 binding to the γ 2 subunit is similarly phospho-dependent. Tyrosine phosphorylation of Y365/367 within the intracellular domain of γ 2 decreased AP2 binding and increased the surface expression of γ 2-containing GABA_AR subtypes and a subsequent increase in IPSC amplitude (80).

Phosphorylation of T375 of the α 1 subunit reduces the affinity to gephyrin which leads to reduced synaptic clustering and a reduction in the amplitude of mIPSCs as these receptors are now free to diffuse away from the synaptic site (101).

Trafficking of extrasynaptic GABA_ARs that mediate tonic inhibition is also sensitive to phosphorylation. PKC phosphorylation of the β 2 subunit (at S410) reduced tonic GABA_AR-mediated inhibition in thalamic and hippocampal neurons (102). Increasing neuronal activity activates L-type voltage-gated Ca^{2+} channels. The rise in Ca^{2+} current stimulates Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) activity which phosphorylates S283 of the GABA_AR β 3 subunit. The net result is a rapid (within 2 min of L-type voltage-gated Ca^{2+} channel activation) and prolonged increase in GABA_ARs on the surface membrane and an increased tonic inhibition (98). The restriction of α 5 subunit containing GABA_ARs to extrasynaptic sites through binding to radixin can be overcome by an activity-dependent dephosphorylation of radixin which releases α 5 subunit containing GABA_ARs resulting in their increased synaptic re-location (74).

PKC-mediated phosphorylation regulates the cell surface expression of extrasynaptic α 4-containing GABA_ARs through increasing the rate of insertion of α 4 to the cell surface. *In vitro* experiments revealed that α 4 subunits are phosphorylated in a PKC-dependent manner at serine 443 within the intracellular loop of α 4 and phorbol ester activation of PKC increased α 4 cell surface insertion (103). In addition, treatment with the neurosteroid THDOC increased the PKC-mediated membrane insertion of the extrasynaptic α 4 subunit and enhanced tonic conduction. This phosphorylation change is independent of the ability of neurosteroids to allosteric potentiate the GABA-evoked current (13,103). When GABA_ARs are comprised of α 4 and β 3 subunits the combined effect of PKC phosphorylation is

increased membrane insertion ($\alpha 4$ subunit effect) and membrane stability ($\beta 3$ subunit effect) producing a rapid and prolonged increase in tonic current.

Furthermore, when S408/9 of $\beta 3$ subunits have been mutated to alanines (S408/409A), the increase in $\alpha 4$ PKC-mediated phosphorylation is abolished and THDOC fails to increase the cell surface expression of the $\alpha 4$ subunit (104).

Conclusions

The amazing control and coordinated placement of GABA_ARs into synaptic and extrasynaptic sites allows for the specific types of GABA_ARs to be located where they mediate phasic and tonic inhibitory currents. Phosphorylation allows for changes in trafficking, in the rate of insertion or endocytosis, and the localization of particular subunits. Normal changes in neuronal activity and pathological conditions such as seizures (96) and inflammation (8) can alter phosphorylation and impact the trafficking of GABA_ARs. Changes in trafficking could have a significant impact on anesthetic endpoints. For example, a PKC-mediated increase in extrasynaptic receptor insertion could increase the sensitivity of anesthetics to bring about loss of consciousness (6). Controlling receptor trafficking could provide a valuable therapeutic target to boost or reduce the level and efficacy of inhibition.

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Figure 1. Time line for GABA_AR recycling

GABA_AR are inserted into the membrane at extrasynaptic sites. From there the receptors diffuse in the plasma membrane. At synaptic sites GABA_ARs are constrained by scaffolding proteins which significantly reduce their diffusion away from synaptic sites. Endocytosis occurs at extrasynaptic sites via mechanisms dependent on the formation of clathrin-coated vesicles. Specific motifs within the intracellular loop of the β and γ subunits bind with the μ subunit of the clathrin adaptor protein AP2 protein to bring about endocytosis. Phosphorylation of residues within the intracellular domains of $\beta 3$ and $\gamma 2$ subunits (by kinases PKC/PKA or Fyn/Src, respectively) interferes with this interaction and therefore stabilizes GABA_ARs in the cell membrane. Once endocytosed, GABA_ARs can undergo ubiquitination and degradation within lysosomes or recycled for reinsertion back to the cell surface.