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Phosphorylation of $GABA_A$ receptors influences receptor trafficking and neurosteroid actions

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Abstract

Rationale—Gamma-aminobutyric acid type A receptors ($GABA_A$Rs) are the principal mediators of inhibitory transmission in the mammalian central nervous system. $GABA_A$Rs can be localized at postsynaptic inhibitory specializations or at extrasynaptic sites. While synaptic $GABA_A$Rs are activated transiently following the release of GABA from presynaptic vesicles, extrasynaptic $GABA_A$Rs are typically activated continuously by ambient GABA concentrations and thus mediate tonic inhibition. The tonic inhibitory currents mediated by extrasynaptic $GABA_A$Rs control neuronal excitability and the strength of synaptic transmission. However, the mechanisms by which neurons control the functional properties of extrasynaptic $GABA_A$Rs had not yet been explored.

Objectives—We review $GABA_A$Rs, how they are assembled and trafficked, the role phosphorylation has on receptor insertion and membrane stabilization. Finally, we review the modulation of $GABA_A$Rs by neurosteroids and how $GABA_A$R phosphorylation can influence the actions of neurosteroids.

Conclusions—Trafficking and stability of functional channels to the membrane surface is critical for inhibitory efficacy. Phosphorylation of residues within $GABA_A$R subunits plays an essential role in the assembly, trafficking and cell surface stability of $GABA_A$Rs. Neurosteroids are produced in the brain and are highly efficacious allosteric modulators of $GABA_A$R mediated current. This allosteric modulation by neurosteroids is influenced by the phosphorylated state of the $GABA_A$R which is subunit dependent, adding temporal and regional variability to the neurosteroid response. Possible links between neurosteroid actions, phosphorylation, and $GABA_A$R trafficking remain to be explored but potential novel therapeutic targets may exist for numerous neurological and psychological disorders which are linked to fluctuations in neurosteroid levels and $GABA_A$ subunit expression.

Keywords

GABA; $GABA_A$ receptors; extrasynaptic receptors; neurosteroids; receptor trafficking; phosphorylation

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Introduction

As the major source of inhibition in the adult brain $GABA_AR$ modulation has an important influence of circuit properties and in neurological conditions. $GABA_ARs$ are located at synaptic sites where they produce phasic inhibition and at extrasynaptic sites where they mediate tonic inhibition of the neuron. The $GABA_ARs$ located at these different sites are formed by different subunits endowing diverse properties to the $GABA_ARs$ allowing them to perform distinctive tasks. Although neurosteroids enhance both phasic and tonic inhibition it appears that extrasynaptic receptors are particularly sensitive to neurosteroids. It has been known that phosphorylation of synaptic $GABA_ARs$ influences trafficking. Little is known about phosphorylation and trafficking of extrasynaptic $GABA_ARs$. In addition, previous data have shown that the modulation of $GABA_ARs$ by neurosteroids is also influenced by phosphorylation.

GABA: Main mediator of Neuronal Inhibition in the CNS

The neurotransmitter γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA is synthesized in the brain from glutamate through the action of the enzyme L-glutamic acid decarboxylase (GAD), which catalyzes the decarboxylation of glutamic acid to form GABA (Erlander et al. 1991). In mammals, GAD exists as two isoforms: GAD65 and GAD67 (with molecular weights of 65 and 67kDa respectively). These two isoforms are encoded by different genes and have a distinct regional distribution throughout the brain (Erlander et al. 1991, Sheik et al.1999). GAD65 is membrane-bound and primarily responsible for vesicular GABA production, whereas GAD67 is located in the cytoplasm and is responsible for cytoplasmic GABA production (Erlander et al. 1991).

After release from pre-synaptic vesicles, GABA is rapidly removed from the synaptic cleft by specialized membrane-bound transporters. GABA uptake is a sodium- and chloride-dependant process mediated by a group of genetically related GABA transporters, GAT-1 to GAT-4 (Liu et al. 1993). In the mammalian brain, GABA uptake is primarily mediated by action of GAT-1 (Minelli et al. 1995). GAT-1 is localized in GABAergic axons and nerve terminals and can also be expressed in glial cells (Minelli et al. 1995). The inhibitory effects of GABA are mediated by two main classes of GABA receptors: Metabotropic G-coupled receptors (GABA type B receptors-$GABA_BR$) and GABA-gated chloride ion channels (GABA type A receptors-$GABA_AR$).

$GABA_AR$ mediate the fast inhibitory actions of GABA

$GABA_ARs$ are the main mediators of fast synaptic inhibition in the CNS. $GABA_ARs$ are chloride permeable channels (Figure 1) that belong to the cys loop ligand gated ion channel super family. Members of this family include nicotinic acetylcholine receptors (nAChRs), glycine receptors, the serotonin (5-hydroxytryptamine) 5-HT3 receptor and the zinc-activated channel (ZAC) (Connolly and Wafford 2004). For this ion channel superfamily, ligand binding is followed by a change in conformation of the channel protein that allows a net inward or outward flow of ions through the membrane-spanning pore of the channel, depending on the electrochemical gradient of the ion. During early developmental stages,

$GABA_A$Rs are primarily depolarizing due to the high intracellular chloride concentrations compared to extracellular chloride levels. In the adult brain, the intracellular chloride concentration is lower compared to the extracellular chloride levels. Therefore in mature neurons, $GABA_A$Rs are generally hyperpolarizing (Rivera et al. 2005). This chloride gradient is maintained primarily by the activity of the $K+/Cl^-$ cotransporter 2 (KCC2). In the majority of adult neurons, activation of $GABA_A$Rs results in a rapid chloride ion influx that results in the hyperpolarization of the cell membrane and thus a reduction in the probability for an action potential to be generated. Therefore, $GABA_A$Rs play a pivotal role in regulating cellular and network excitability in the CNS, which underlies all physiological and behavioral processes.

The members of cys-loop family form heteropentamers assembled from a wide range of heterologous subunits. To date, 19 $GABA_A$R subunits have been identified. These subunits are divided into eight classes according to sequence homology; α1–6, β1–3, γ1–3, δ, ε, θ, ρ(1–3), and π (Olsen and Sieghart 2008; Sieghart and Sperk 2002).

The genomic location of the 19 genes encoding $GABA_A$R subunits has been determined, and 14 of them are arranged in $GABA_A$R gene clusters (Russek 1999). There are gene clusters on chromosomes 4 (α2, α4, β1 and γ1) and 5 (α1, α6, β2 and γ2) as well as on chromosomes 15 (α5, β3 and γ3) and X (α3, θ and ε). The gene for the π subunit is also located on chromosome 5 but at a site distant from the α1, α6 β2 γ2 cluster. Subunits ρ2 and ρ3 are mapped together on chromosome 6, whereas the δ and ρ3 subunits are located on their own on chromosomes 1 and 3 respectively. Each gene cluster contains gene encoding for α, β and γ/ε class (Darlison et al. 2005; Russek 1999). This gene organization has been proposed to be a result of early gene duplication events from a single ancestral αβγ $GABA_A$R subunit cluster (Darlison et al. 2005; Russek 1999) and is believed to help coordinate gene expression (Joyce 2007).

Like all other members of the cys-loop family, all $GABA_A$R subunits share a common structure: they have a large extended amino-terminal extracellular domain containing a cysteine loop signature, four highly conserved transmembrane domains (TMs), and a large intracellular loop domain between TM3 and TM4 (Arancibia-Carcamo and Kittler 2009; Connolly and Wafford 2004) (Figure 1). Using homology modeling based on the structure of the nicotinic acetylcholine receptor (Unwin 1995), it is believed that the lining of the water-filled channel pore is formed by the alignment of TM2 domains when subunits are arranged as a pentameric structure, and they extend through the membrane as alpha-helixes bending outwards (Corringer et al. 2000). TM1, TM3 and TM4 compose the interface with lipids and are thought to isolate TM2 lining from the hydrophobic membrane environment (Connolly and Wafford 2004).

Sites within the extracellular amino-terminal are important for oligomerization of the protein and for subunit-subunit interactions (Arancibia-Carcamo and Kittler 2009; Jacob et al. 2008). The binding site for GABA is located at the interface between α and β subunits (Connolly and Wafford 2004) (Figure 1). The accepted stoichiometry for αβγ and αβδ $GABA_A$Rs is 2:2:1 (Barrera et al. 2008; Farrar et al. 1999; Patel et al. 2014), thus two β/α

subunit interfaces in the pentameric structure occur which form the two GABA binding sites (Olsen and Sieghart 2009).

The intracellular domain is the most genetically divergent part of each individual $GABA_AR$ subunit. This domain is a critical site for cytoplasmic protein interactions with regulatory and signaling molecules including microtubule-binding proteins, cytoskeletal proteins, kinase-anchoring proteins and neurotransmitter transporters (Jacob et al. 2008; Luscher et al. 2011; Luscher and Keller 2004). In addition, the intracellular loop has multiple sites for post-translational modifications such as ubiquitination, palmitoylation and phosphorylation (Jacob et al. 2008; Kittler and Moss 2003). These protein interactions and post-translational modifications modulate receptor activity and trafficking.

Regulation of $GABA_AR$ oligomerization, transport, maturation and trafficking

The heterogeneity of $GABA_AR$ subtypes is restricted primarily by regulation governing the proper assembly of the receptor (Sieghart and Sperk 2002). Although a large number of $GABA_AR$ subtypes could be possible, studies suggest that only a limited number of $GABA_AR$ subunit combinations can oligomerize and reach the neuronal cell membrane (Arancibia-Carcamo and Kittler 2009).

The oligomerization of $GABA_ARs$ occurs in the Endoplasmic Reticulum (ER). This process is mediated by critical assembly-domains located within the amino-terminals of $GABA_AR$ subunits and occurs within five minutes after translation (Gorrie et al. 1997; Jacob et al. 2008; Kittler et al. 2002). Receptors must be assembled and reach conformational maturity in the ER before transport to the plasma membrane. Studies in cell lines have shown that most single subunits are not capable of leaving the ER, and are targeted for proteosomal degradation (Jacob et al. 2008; Kittler et al. 2002; Moss and Smart 2001). Although some subunits (β1, β3 and γ2S) are able to reach the cell membrane, they are unable to form functional GABA-sensitive channels and are rapidly targeted for degradation (Connolly et al. 1999; Davies et al. 1997; Krishek et al. 1996; Sanna et al. 1995).

$GABA_ARs$ are typically composed of two α, two β and one γ or δ subunit (Backus et al. 1993; Barrera et al. 2008; Farrar et al. 1999; Patel et al. 2014). $GABA_ARs$ composed of only α and β subunits can form functional receptors in heterologous cell lines and have been suggested to exist in small numbers within neurons where they may mediate tonic inhibition (Mortensen and Smart 2006). Combinations of αγ and βγ are mostly retained in the ER and when α, β and γ are co-expressed together the formation of αβγ-$GABA_AR$ is strongly favored over receptors composed of only α and β subunits (Angelotti and Macdonald 1993).

The assembly and maturation within the ER are regulated by mechanisms involving ER-resident chaperones including Calnexin and Binding immunoglobulin protein (BiP) (Ehya et al. 2003; Jacob et al. 2008; Sarto-Jackson et al. 2006; Taylor et al. 2000). Calnexin and BiP are involved in regulating quality control of proteins within the secretory pathway (Jacob et al. 2008; Taylor et al. 2000). After oligomerization in the ER, $GABA_ARs$ are then trafficked to the Golgi apparatus to be sorted into vesicles before insertion in the neuronal plasma

membrane. This process is regulated by receptor-associated proteins that interact with the intracellular loop of $GABA_AR$ subunits (Jacob et al. 2008). Plic-1 (Protein that links integrin associated protein with the cytoskeleton-1) is involved in $GABA_AR$ stability in the ER and in the transport of the receptor through the secretory pathway (Figure 2). Plic-1 regulates $GABA_AR$ transport and maturation through the secretory pathway via interaction with all isoforms of α and β subunits (Bedford et al. 2001). The Plic-1 protein contains ubiquitin-like proteasome binding domains as well as ubiquitin-associated domains and is therefore able to inhibit ubiquitin-mediated proteolysis of $GABA_ARs$ (Walters et al. 2002).

When in the Golgi, γ2-containing $GABA_ARs$ undergo palmitolation via interaction of the Golgi-specific DHHC zinc finger protein (GODZ) with cytoplasmic serine residues within the γ2 subunit. GODZ palmitolation of the γ2 subunit is critical for the subsequent accumulation of synaptic $GABA_ARs$ at inhibitory synapses (Rathenberg et al. 2004).

Proper modulation of inhibition through $GABA_ARs$ is dependent on receptor cycling between the cell surface and intracellular compartments (Jacob et al. 2008). To date, a number of regulatory proteins have been implicated in the cycling of $GABA_ARs$ between the cell membrane and intracellular pools. These include BIG2 (Brefeldin-A inhibited GDP/GTP exchange factor 2), GABARAP ($GABA_AR$ associated protein), and components of the endocytotic machinery. BIG2 mediates the $GABA_ARs$ exit from the Golgi towards the cell surface as well as $GABA_AR$ endocytic recycling via interaction with a sequence motif within the intracellular domain of β subunits (Charych et al. 2004). GABARAP is an ubiquitin-like protein that interacts with microtubules and with the intracellular loop of all γ subunits (Leil et al. 2004; Wang et al. 1999). It is involved in the translocation of $GABA_ARs$ from intracellular compartments to the cell membrane (Kittler et al. 2004a; Leil et al. 2004). Other proteins involved in $GABA_AR$ trafficking are Phospholipase C-Related catalytically Inactive Proteins 1 and 2 (PRIP1/2) and N-ethylmaleimide Sensitive Factor (NSF). PRIP1/2 and NSF influence receptor trafficking indirectly through GABARAP and through a direct interaction with receptor subunit. NSF directly interacts with β subunits and can interact with GABARAP to increase receptor trafficking from the Golgi apparatus (Goto et al. 2005). In addition to binding with GABARAP, PRIP's bind directly with β subunits and γ2 subunits, enhancing the trafficking of $GABA_ARs$ to the cell surface (Mizokami et al. 2007; Terunuma et al. 2004; Uji et al. 2002).

$GABA_AR$ endocytosis and recycling

Endocytosis of $GABA_ARs$ occurs primarily via mechanisms dependent on dynamin and clathrin (Figure 3). These processes are mediated by the interaction of the clathrin adaptor protein AP2 with residues located within the cytoplasmic intracellular loop of the β, γ, and δ subunits (Kittler et al. 2005; Kittler et al. 2008; Kittler et al. 2000). Work by Kittler et al. 2005, 2008 identified a ten amino acid motif within the cytoplasmic domain of all β subunits, as critical for mediating the $GABA_AR$ interaction with the μ2 subunit of the AP2 protein.

The interaction of the μ2 subunit with this motif within the β subunit is negatively regulated by phosphorylation of conserved serines within the AP2 binding motif (S408 in β1, S410 in

β2, and S408/409 in β3). That is, AP2 can bind to $GABA_A$Rs and trigger receptor internalization only when this site is dephosphorylated by protein phosphatases PP1α and PP2A (Kittler et al. 2005). The protein PRIP-1, mentioned above for its role in receptor trafficking, also binds to and inactivates PP1α, in so doing PRIP-1 can also prevent receptor internalization by preserving the phosphorylated state of the β subunit (Terunuma et al. 2004). Serines 408 and 409 in the β3 subunit can be phosphorylated by Protein Kinase A (PKA), Protein Kinase C (PKC), Calcium/Calmodulin-dependant Kinase II (CAMKII) and AKT, resulting in an increase in surface levels of β3 subunit containing $GABA_A$Rs (Brandon et al. 2000; Brandon et al. 2002; McDonald et al. 1998; McDonald and Moss 1994).

The AP2 μ2 subunit also interacts with two motifs within the intracellular domain in the γ subunit. One consists of a twelve amino acid domain analogous to the AP2 binding site in the β subunit (Smith et al. 2008). The second is a high affinity γ-specific site YGYECL motif that contains a phosphorylation site at tyrosines 365/367 (Kittler et al. 2008; Smith et al. 2008). These residues can be phosphorylated by Fyn kinase and other members of the Src-family of tyrosine kinases (Jurd et al. 2010) and the phosphorylation of these sites interferes with AP2 binding and hence prevents $GABA_A$R internalization (Boehm et al. 2004). The intracellular domain of the δ subunit also binds the AP2 μ2 subunit (Kittler et al. 2005). Recently, it has been demonstrated that the observed downregulation of extrasynaptic $GABA_A$Rs in the hippocampus and dentate gyrus following ethanol administration is partly due to increased AP2 μ2 binding to the $GABA_A$R δ subunit leading to receptor endocytosis (Gonzalez et al. 2012).

If $GABA_A$Rs were to be internalized, once they are in endosomes $GABA_A$Rs can become ubiquitinated and targeted for lysosomal degradation (Arancibia-Carcamo et al. 2009). Alternatively, regulatory proteins such as the Huntingtin-associated protein 1 (HAP1) (Kittler et al. 2004b; Twelvetrees et al. 2010) and the Calcium Modulating Cyclophilin Ligand (CAML) (Yuan et al. 2008) can interact with the cytoplasmic domains of the β and γ subunits respectively, and in this way facilitate vesicular transport and recycling back to the cell membrane.

We have recently shown that the cell surface expression of the α4 subunit is also modified by phosphorylation. The α4 subunit is phosphorylated by PKC within the large intracellular domain at serine 443, a covalent modification that increases cell surface stability of receptors containing α4 subunits by promoting their transport from the ER to the cell surface (Abramian et al. 2010).

Subcellular localization of $GABA_A$R subtypes

The heterogeneity of $GABA_A$R subunit composition is also carefully regulated by regional and temporal specificity in the patterns of expression in the CNS. This heterogeneity allows for $GABA_A$Rs with different physiological and pharmacological properties as well as differential expression throughout the brain (Rudolph et al. 2001; Sieghart and Sperk 2002). $GABA_A$Rs of different subunit composition have different subcellular localization (Connolly et al. 1996). $GABA_A$Rs composed of the α1–3, β1–3 and γ2 are predominantly

located at synaptic sites (Rudolph and Möhler 2006), whereas $GABA_A$Rs composed of the α4–6, β2/3 and δ subunits are primarily localized at sites distant from synapses or extrasynaptic sites (Chandra et al. 2006; Farrant and Nusser 2005; Zheleznova et al. 2009).

The cell membrane distribution of synaptic and extrasynaptic $GABA_A$Rs is dynamically regulated via the interactions with sub-synaptic scaffold molecules. During synaptic $GABA_A$R membrane targeting, receptors are first introduced to the neuronal membrane at extrasynaptic sites, followed by subsequent lateral membrane diffusion to synaptic sites (Bogdanov et al. 2006).

Scaffold proteins located at post-synaptic localizations bind and cluster surface synaptic $GABA_A$Rs at sites directly opposite GABA releasing axon terminals (Jacob et al. 2008). Gephyrin is the principal intracellular scaffolding molecule for both glycinergic and GABAergic synapses and has a critical role in regulating the clustering of synaptic $GABA_A$Rs at inhibitory synapses (Fritschy et al. 2008; Levi et al. 2004; Mukherjee et al. 2011). Gephyrin interacts with proteins that regulate microfilament dynamics (such as profiling I and II) and with microtubules resulting in the formation of a hexagonal protein lattice that allows for the organization of synaptic $GABA_A$Rs distribution in the cell membrane (Kirsch et al. 1995; Luscher et al. 2011; Mammoto et al. 1998). Synaptic adhesion molecules play an important role in the maturation and stabilization of inhibitory synapses (Cheng et al. 2006; Jamain et al. 2008; Ullrich et al. 1995). Tran-synaptic complexes of pre-synaptic β-neurexins and post-synaptic Neuroligin 2 (NL2) contribute to the proper alignment of pre- and post- synaptic molecules and structural maturation of inhibitory synapses (Panzanelli et al. 2011).

The cytoskeletal interactions that involve the anchoring at specific membrane localizations for those $GABA_A$R located at extrasynaptic sites are less understood than for synaptic receptors. The cytoskeletal protein Radixin (a member of the ERM-ezrin, radixin and moesin family) has been implicated in the actin cytoskeleton anchoring of the predominantly extrasynaptic α5 subunit (Loebrich et al. 2006). However, the functional relevance of α5-containing $GABA_A$R clustering at extrasynaptic sites is unknown. The expression of a dominant-negative radixin construct in neurons abolishes the membrane clustering of α5-containing $GABA_A$R but has no effect on GABA- mediated currents (Loebrich et al. 2006).

Synaptic $GABA_A$Rs mediate phasic inhibition

$GABA_A$Rs located at synaptic and extrasynaptic sites are activated in a different manner and mediate distinct forms of inhibition. Synaptic $GABA_A$Rs are activated in a transient manner after brief exposure to high concentrations of GABA released from the presynaptic membrane. This transient activation of synaptic $GABA_A$Rs results in phasic inhibition (Jacob et al. 2008). Activation of synaptic $GABA_A$Rs results in a rapid chloride ion influx that creates a transient or phasic, but significant reduction in the probability for an action potential to be generated due to rapid hyperpolarization of the plasma membrane.

Extrasynaptic $GABA_A$Rs mediate tonic inhibition

Extrasynaptic $GABA_A$Rs can be activated in a persistent, less temporally restricted manner by low ambient concentrations of GABA that either escapes from the synapse into the extracellular space or is released from non-synaptic sites such as neurogilaform cells and astrocytes (Kozlov et al. 2006; Lee et al. 2010; Olah et al. 2009). Recently it has been noted that tonic current can originate from some $GABA_A$Rs that have a higher probability to transition from a closed to an open state in the absence of GABA (Wlodarczyk et al. 2013). When activated, extrasynaptic $GABA_A$Rs generate an uninterrupted form of conductance that is referred to as tonic inhibition which results in a persistent reduction in the cell's input resistance. This results in a reduction in both the size and duration of excitatory post-synaptic potentials and will in turn reduce the temporal and spatial frame for synaptic integration. Ultimately, tonic inhibition persistently reduces the likelihood for an action potential to occur.

Tonic currents mediated by extrasynaptic $GABA_A$Rs have been described in various brain regions including in layer III cells of the somatosensory cortex (Salin and Prince 1996), cerebellar and dentate granule cells (Brickley et al. 1996; Nusser and Mody 2002), hippocampal interneurons and pyramidal cells (Bai et al. 2001; Semyanov et al. 2003), embryonic and developing neurons (Ge et al. 2006), neocortical layer 2/3 pyramidal cells (Drasbek et al. 2007; Drasbek and Jensen 2006), the spinal cord (Cronin et al. 2004), and in several sub cortical structures including hypothalamic (Sarkar et al. 2011; Sergeeva et al. 2005), thalamocortical neurons (Belelli et al. 2005; Cope et al. 2005) and medium spiny neurons of the striatum (Ade et al. 2008; Santhakumar et al. 2010).

The fact that $GABA_A$R-mediated tonic inhibition occurs in many brain regions, changes during different developmental stages (Ge et al. 2006; LoTurco et al. 1995) and exhibits cell-type specific differences in magnitude suggests critical physiological roles for tonic inhibition. Indeed, tonic inhibition appears to be essential for the control of firing frequency (Rossi et al. 2003), for modulation of the firing threshold (offset), and the gain of transmission (Hamann et al. 2002; Mitchell and Silver 2003; Pavlov et al. 2009), all of which ultimately modulate network excitability (Farrant and Nusser 2005; Vida et al. 2006). During development, depolarization mediated by tonic currents has a critical role in neuronal migration, dendritic arborization, and the formation of synapses (Ge et al. 2006; Manent et al. 2005).

Extrasynaptic $GABA_A$R properties

Extrasynaptic $GABA_A$Rs exhibit different pharmacological properties from their synaptic counterparts; they are largely insensitive to benzodiazepines (Cope et al. 2005; Nusser and Mody 2002) and they are highly sensitive to THIP (4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol/Gaboxadol) a selective $GABA_A$R agonist (Brown et al. 2002; Wohlfarth et al. 2002). Low concentrations of the $GABA_A$R antagonist Gabazine (SR95531) abolish inhibitory post synaptic currents while having a small or no effect on $GABA_A$R-mediated tonic conductance (Semyanov et al. 2003; Stell and Mody 2002). Furthermore, extrasynaptic $GABA_A$Rs are targets for, and principal mediators of response to, various endogenous and

exogenous molecules including anticonvulsants, anesthetics and neurosteroids (Belelli et al. 2002; Bianchi and Macdonald 2003; Kretschmannova et al. 2013; Stell et al. 2003).

Extrasynaptic $GABA_A$Rs are uniquely sensitive to neurosteroids

Neuroactive steroids can originate as metabolites of systemically produced progesterone or deoxycorticosterone, but can also be synthesized *de novo* in the brain by neurons and glia (Belelli and Herd 2003; Compagnone and Mellon 2000; Maguire and Mody 2007). The enzymes and steroid mitochondrial transporters necessary for de novo synthesis of pregnane neuorosteroids are present in many CNS regions (Mellon and Vaudry 2001). The P450cc mitochondrial cholesterol side-chain cleavage enzyme (P450cc) catalyzes the rate limiting step in *de novo* neurosteroid synthesis in which cholesterol is converted to pregnenolone (Mellon and Vaudry 2001). In addition, the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase, which are required for the synthesis of 3α-hydroxy-5α-pregnane-20-one/ Allopregnanolone (THPROG- from progesterone) and 3α,5α-3,21-dihydroxypregnan-20-one/Tetrahydrodeoxycorticosterone (THDOC- from deoxycorticosterone), have been shown to be expressed in the brain in a region and cell-type specific manner (Agis-Balboa et al. 2006). Unlike classical steroids, which act via their nuclear receptors to regulate gene expression, neurosteroids rapidly alter neuronal excitability via non-genomic mechanisms. Pregnane steroids containing a 3-α hydroxy ring have been shown to be potent steroselective allosteric modulators of $GABA_A$Rs, having anxiolytic, anticonvulsant sedative and anesthetic effects (Majewska 1992; Paul and Purdy 1992).

Extrasynaptic $GABA_A$Rs containing the δ are the most sensitive to neurosteroid modulation (Belelli et al. 2002; Bianchi and Macdonald 2003; Stell et al. 2003). Low physiological concentrations (10–100nM) of 3α,5α-THDOC greatly enhance the tonic conductance mediated by extrasynaptic $GABA_A$Rs with little or no effect on the phasic conductance mediated by synaptic $GABA_A$Rs, in both dentate gyrus and cerebellar granule cells (Stell et al. 2003). At the single channel level, neurosteroids increase the open duration and the frequency of $GABA_A$R channel openings with no effect on the single channel conductance (Callachan et al. 1987; Twyman and Macdonald 1992).

Neurosteroids enhance $GABA_A$Rs via a distinct binding pocket

The stereoselectivity of the potent interaction between neurosteroids and native $GABA_A$Rs strongly suggested early on the possibility of a neurosteroid modulatory site on the receptor protein. Electrophysiological and radioligand binding experiments provided evidence that the modulatory site for neurosteroids on $GABA_A$Rs was distinct from the binding site for benzodiazepines and other known allosteric recognition modulators (Callachan et al. 1987; Peters et al. 1988). Subsequent homology modeling studies coupled to the use of $GABA_A$R chimeras between steroid insensitive *Drosophila*-RDL subunits and α subunits led to the identification of critical residues for neurosteroid modulation. These studies revealed the importance of residues with side chains that could form hydrogen bond interactions with neurosteroid molecules. The important residues are conserved among all α subunit isoforms and are glutamine (Q) located within TM1 and the residues asparagine and tyrosine located within TM4 (Hosie et al. 2006; Hosie et al. 2007). Mutating one such site in the TM1 region

of the α4 subunit (Q241L) significantly abolished THDOC enhancement of the receptor (Hosie et al. 2009). Furthermore, they provided evidence that the δ subunit does not contribute to the neurosteroid modulation site and is likely to be regulating the efficacy of neurosteroid potentiation after the initial binding to the $GABA_AR$ (Hosie et al. 2009).

Tonic inhibition, Neurosteroids and Disease

Disturbances in the tonic inhibition mediated by extrasynaptic $GABA_ARs$ has been observed in a wide range of psychiatric and neurological conditions including some forms of epilepsy, addiction, cognitive impairments, sleep disorders, anxiety disorders, post-partum depression and stress-related disorders (Belelli et al. 2009; Kato et al. 2007; Maguire and Mody 2008; Maguire et al. 2005; Maldonado-Aviles et al. 2009; Nie et al. 2011; Uhlhaas and Singer 2010). Mutations within the δ and other extrasynaptic $GABA_AR$ receptor subunits have been implicated in some forms of epilepsy (Macdonald et al. 2010), childhood onset of some mood disorders (Feng et al. 2010), and schizophrenia (Maldonado-Aviles et al. 2009).

Many of these disorders also involve changes in the levels of neurosteroids, that occur following physiological changes in the levels of ovarian and adrenal cortex hormones. For example, catamenial epilepsy (a form of epilepsy in which female patients show a cyclic variation in seizure intensity depending on the menstrual cycle phase) has been linked to changes in tonic inhibition during the ovarian cycle (Maguire et al. 2005). Ganaxolone (an analogue to the neurosteroid allopregnanolone) is currently being used in clinical trials for the treatment of this disorder and has been shown to be an effective anticonvulsant in an animal model (Bialer et al. 2013). Anxiety linked to premenstrual dysmorphic disorder (PMDD) has been associated with neurosteroid-mediated changes in tonic inhibition in animal models (Maguire et al. 2005; Smith et al. 1998).

Work by Maguire and Moody (2008), linked discrepancies in the number of δ-containing extrasynaptic $GABA_ARs$ and the neurosteroids allopregnanolone (progesterone metabolite) with post-partum depression. Elevated allopregnanolone levels during pregnancy (as a direct result of the 100-fold increase in progesterone during this period) results in a compensatory reduction in the number of δ-containing $GABA_ARs$. In a mouse model, delay in restoring δ-containing GABAARs number to prepregnancy levels, resulted in a severe depression-like phenotype (Maguire and Mody 2008).

Of particular relevance for stress-related disorders, a recent study has shown that corticotropin-releasing hormone (CRH) neurons of the hypothalamus are modulated by the stress hormone metabolite THDOC through its actions on δ-containing $GABA_ARs$. CRH neurons are a critical component of the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the physiological response to stress. Under normal physiological conditions, THDOC decreased HPA axis activity by potentiating the inhibitory effects of GABA on CRH neurons. However, during acute stress conditions THDOC activates the HPA axis. This is due to a collapse in the chloride gradient that occurs following the dephosphorylation of KCC2. THDOC actions on δ-containing $GABA_ARs$ seem to constitute a positive feedback mechanism onto CRH neurons, which is required for the proper physiological response to stress (Sarkar et al. 2011).

Neurosteroids regulate changes in $GABA_AR$ subunit expression

Neurosteroids dynamically regulate changes in $GABA_AR$ subunit expression. For example, the δ and α4 subunits have been shown to undergo marked changes in expression in response to fluctuating steroid levels (Gulinello et al. 2001; Hsu et al. 2003; Maguire and Mody 2007; 2009). The levels of steroid hormones can fluctuate in a wide range of physiological states including stress, puberty, pregnancy and the menstrual cycle. Steroid-induced fluctuations in $GABA_AR$ subunit expression result in alterations in neuronal excitability and are implicated in many neurological disorders. However, the molecular mechanisms by which neurosteroids alter $GABA_ARs$ subunit expression and function are not well understood.

For synaptic $GABA_ARs$, the trafficking mechanisms and protein interactions that regulate receptor cell surface localization are essential for the changes in synaptic strength mediated by GABA receptor signaling (Jacob et al. 2008). Endocytosis is a critical process for the regulation of the number of surface synaptic neurotransmitter receptors. Our laboratory has shown that phosphorylation of residues within synaptic $GABA_AR$ subunits regulates the interaction of $GABA_AR$ subunits with protein complexes that mediate endocytosis (Jacob et al. 2008; Luscher et al. 2011). Whether extrasynaptic $GABA_AR$ cell surface expression is modulated in a similar manner is not fully understood but PKC-mediated phosphorylation of the α4 subunit increases membrane surface expression of α4β3 subunit containing $GABA_ARs$ in recombinant cells and in the dentate gyrus region of the hippocampus where α4/δ subunit containing $GABA_ARs$ form extrasynaptic receptors (Abramian et al. 2010). Next, we explore how phosphorylation of $GABA_AR$ subunits influence the actions of neurosteroids and discuss a possible link between neurosteroids and subunit phosphorylation leading to changes in $GABA_AR$ expression.

Phosphorylation and neurosteroid action

Neurosteroids have been shown to be potent positive allosteric modulators of $GABA_ARs$ and are particular efficacious on extrasynaptic $GABA_ARs$. Previous experiments have suggested that PKC activity enhances neurosteroid-mediated modulation of $GABA_ARs$, or PKC activity is required for such modulation (Fancsik et al. 2000; Harney et al. 2003; Leidenheimer and Chapell 1997). Neurosteroid modulation of IPSCs in hippocampal CA1 pyramidal neurons is dependent on both PKA and PKC, whereas in the dentate gyrus only stimulation of PKC was effective in enhancing neurosteroid effects (Harney et al. 2003). Contrary to this PKC-dependent enhancement by neurosteroids, Kia et al (2011) reported that in piriform cortex pyramidal neurons, PKC activation with the phorbal ester, PMA reduced THDOC modulation of phasic and tonic GABA-mediated currents and suggested that different isomers of PKC phosphorylated synaptic and extrasynaptic $GABA_ARs$ (Kia et al. 2011). Region specific effects are likely to be observed based on region dependent expression of different subunits and kinase activity.

In addition to the well characterized positive allosteric modulation of $GABA_ARs$, neurosteroids have been demonstrated to affect subunit expression, particularly the α4 and δ subunitys. Because phosphorylation of receptor subunits greatly influences the membrane

expression of $GABA_A$Rs, and that we have previously found that the α4 subunit can be phosphorylated by PKC at serine 443 which results in an increase in membrane expression of α4 subunit containing $GABA_A$Rs (Abramian et al. 2010), we examined if neurosteroids could influence membrane expression by changing the phosphorylated state of subunits. We demonstrated in α4 expressing recombinant cells, neurons in culture, and in brain slices that neurosteroids enhance the PKC phosphorylation of S443 within α4 subunits, and this neurosteroid-mediated effect is independent of the allosteric actions. PKC-mediated phosphorylation of α4 subunits enhances the insertion of α4 subunit containing $GABA_A$R subtypes into the membrane, resulting in a selective and sustained elevation in the efficacy of tonic inhibition (Abramian et al. 2014). The exact mechanism behind this phospho-dependent membrane insertion of α4 subunit containing $GABA_A$Rs has yet to be determined but represents a novel mechanism by which neurosteroids can alter neuronal inhibition.

Summary

Receptor expression is critical for maintaining a healthy excitation / inhibition balance. Phosphorylation of $GABA_A$R subunits is a critical component in subunit trafficking and membrane stability of receptors. In some areas of the brain, the allosteric modulation of $GABA_A$R-mediated currents by neurosteroids is dependent upon phosphorylation. In addition to their allosteric modulation of $GABA_A$Rs, neurosteroids can exert long-term effects on neuronal excitation by dynamically regulating the expression levels of extrasynaptic $GABA_A$Rs through a phosphorylation-dependent mechanism. Dysregulation of neurosteroid signaling is associated with premenstrual dysphoric disorder, panic disorder, depression, schizophrenia and bipolar disorder. Determining the mechanism by which neurosteroids modify $GABA_A$R expression may provide novel therapeutic strategies for these conditions.

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Abbreviations

AP2	clathin adaptor protein 2
BIG2	Brefeldin-A inhibited GDP/GTP exchange factor 2
CAML	Calcium Modulating Cyclophilin Ligand
ER	Endoplasmic Reticulum
GABA	γ-aminobutyric acid
$GABA_A$Rs	γ-aminobutyric acid type A receptors
GABARAP	$GABA_A$R associated protein
GODZ	Golgi-specific DHHC zinc finger protein

HAP1	Huntingtin-associated protein 1
PKC	Protein Kinase C
THDOC	Allotetrahydrodeoxycorticosterone
THIP	4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol

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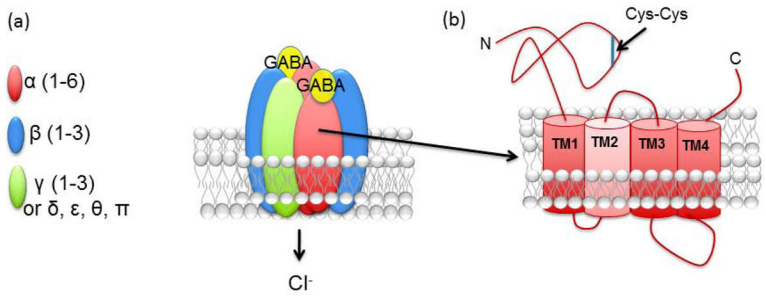

Fig. 1. Structure of $GABA_AR$

$GABA_AR$ are composed of five subunits assembled as a heteropentamer. **(A)** The prototypical $GABA_AR$ is composed of two α, two β, and one γ or δ. 19 $GABA_AR$ subunits have been identified to date and they are divided into seven groups on the basis of sequence similarity. **(B)** The common molecular $GABA_AR$ subunit structure is composed of a large N-terminal extracellular domain, four transmembrane domains, a short C-terminal extracellular domain and a long intracellular loop domain between TM3 and TM4. The intracellular domain contains critical sites for posttranslational modifications and protein-protein interactions that modulate receptor activity.

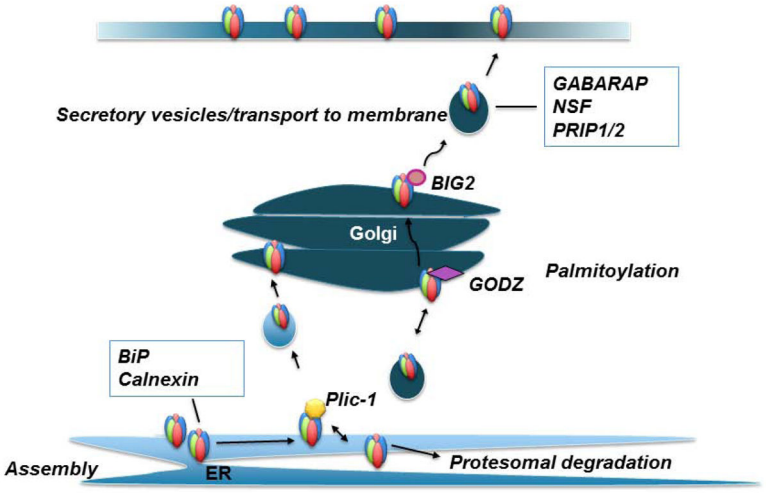

Fig. 2. Assembly, maturation and trafficking of $GABA_AR$s

$GABA_AR$s are assembled as pentamers in the ER. This process is primarily regulated by the interaction of the ER-chaperones BiP and calnexin with residues located in the extracellular domain of $GABA_AR$ subunits. Improperly folded and unassembled $GABA_AR$ subunits are targeted for ubiquitination/proteosomal degradation. The ubiquitin-like protein Plic-1 inhibits degradation via interaction with sites located within the intracellular domain of α and β subunits. Plic-1 promotes $GABA_AR$ transport from the ER to the Golgi apparatus. In the Golgi, the palmitoytransferase GODZ palmitoylates a cysteine residue located within the intracellular loop of the γ subunit. Palmitoylation promotes $GABA_AR$ transport through the Golgi towards inhibitory synapses in the cell membrane. The translocation of $GABA_AR$ from the Golgi to the cell surface is believed to be mediated via BIG2 interaction with the intracellular domain of β subunits as well as other regulatory proteins including GABARAP and NSF.

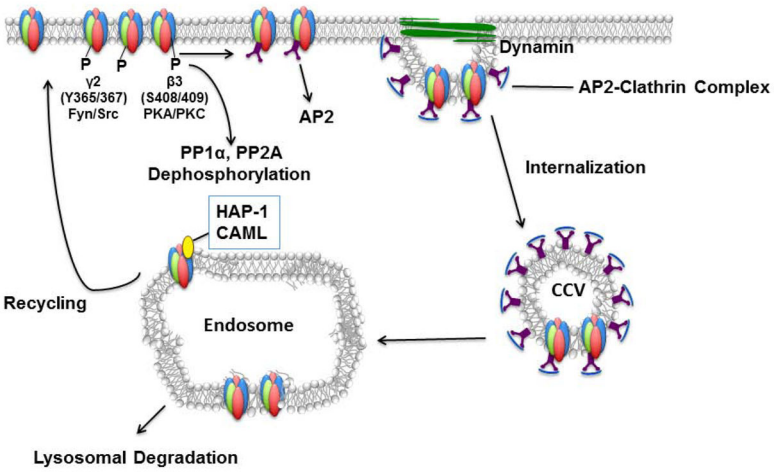

Fig. 3. $GABA_AR$ endocytosis and recycling

$GABA_AR$ endocytosis occurs primarily via mechanisms dependent on the formation of clathrin-coated vesicles. Clathrin- and dynamin-mediated endocytosis is regulated in a phospho-dependent manner via the interaction of specific motifs within the intracellular loop of the β and γ subunits with the μ subunit of the AP2 protein. Phosphorylation of residues within the intracellular domains of β3 and γ2 subunits (by kinases PKC/PKA or Fyn/Src, respectively) interferes with this interaction and therefore stabilizes $GABA_ARs$ in the cell membrane. When endocytosed, $GABA_ARs$ can be ubiquitinated and degraded via lysosomal degradation. Alternatively, the receptors can interact with regulatory lysosomal proteins such as HAP1, and CAML, which promotes the transport and recycling of receptors back to the cell surface (Arancibia-Cárcamo et al., 2009).