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Review

$GABA_A$ Receptor Trafficking-Mediated Plasticity of Inhibitory Synapses

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Proper developmental, neural cell-type-specific, and activity-dependent regulation of GABAergic transmission is essential for virtually all aspects of CNS function. The number of $GABA_A$ receptors in the postsynaptic membrane directly controls the efficacy of GABAergic synaptic transmission. Thus, regulated trafficking of $GABA_A$ receptors is essential for understanding brain function in both health and disease. Here we summarize recent progress in the understanding of mechanisms that allow dynamic adaptation of cell surface expression and postsynaptic accumulation and function of $GABA_A$ receptors. This includes activity-dependent and cell-type-specific changes in subunit gene expression, assembly of subunits into receptors, as well as exocytosis, endocytic recycling, diffusion dynamics, and degradation of $GABA_A$ receptors. In particular, we focus on the roles of receptor-interacting proteins, scaffold proteins, synaptic adhesion proteins, and enzymes that regulate the trafficking and function of receptors and associated proteins. In addition, we review neuropeptide signaling pathways that affect neural excitability through changes in $GABA_A$R trafficking.

Introduction

Inhibitory neurotransmission in the brain is largely mediated by γ-aminobutyric acid (GABA) acting through GABA type A receptors ($GABA_A$Rs). These receptors are heteropentameric GABA-gated chloride channels that belong to the Cys-loop ligand-gated ion channel superfamily (Figure 1A) (Barnard et al., 1998). In addition to fast actions of GABA via $GABA_A$Rs, GABA also modulates neural activity on a slower time scale through activation of $GABA_B$Rs belonging to the G protein-coupled receptor superfamily. $GABA_A$Rs are expressed ubiquitously in neurons along the entire neuraxis. Dynamic changes in their expression and function accordingly are implicated in the regulation of virtually all aspects of brain function. In addition, $GABA_A$R activity controls important aspects of brain development, including proliferation and differentiation of neural progenitors, neural migration, and dendritic maturation of neurons. Deficits in $GABA_A$R-mediated GABAergic transmission are implicated in the etiology of epilepsy (Fritschy, 2008), anxiety disorders (Lydiard, 2003), mood disorders (Craddock et al., 2010; Luscher et al., 2011), and schizophrenia (Charych et al., 2009). A detailed understanding of the mechanisms that regulate functional expression of $GABA_A$Rs at synapses therefore is a prerequisite for an understanding of the causes of these disorders.

Experimental evidence indicates that synaptically released neurotransmitters saturate their receptors (Clements, 1996) and hence, that the functional strength of GABAergic synapses changes in proportion with the number of postsynaptic $GABA_A$Rs (Otis et al., 1994; Nusser et al., 1997). Consistent with this idea, even modest reductions in postsynaptic $GABA_A$Rs (5%–35%) in $GABA_A$R mutant mice have significant behavioral consequences (Crestani et al., 1999; Shen et al., 2010b). The focus of this review is on mechanisms that underlie dynamic changes in the posttranslational biogenesis, surface accumulation, turnover, and trafficking of $GABA_A$Rs, which arguably represent the most important and diverse biological means to adjust GABAergic transmission. First, we will provide brief overviews of the structure-function relationships of different $GABA_A$R subtypes and the different modes of regulation of postsynaptic GABAergic function. We will then summarize current understanding of the processes that regulate the assembly of subunits into transport-competent $GABA_A$Rs, the exocytosis of receptors to the plasma membrane, and the endocytic recycling and degradation of $GABA_A$Rs. Next we will focus on mechanisms that regulate the differential distribution of $GABA_A$Rs at the cell surface between synaptic and extrasynaptic membrane sites, the interaction with postsynaptic protein scaffold and adhesion molecules, and dynamic changes in surface mobility of $GABA_A$Rs. Finally, we discuss neuropeptide signaling systems that act upstream of $GABA_A$Rs and exert their neural effects in part through altered $GABA_A$R trafficking.

Structure of $GABA_A$Rs in Relation to Cellular Distribution and Function

$GABA_A$Rs are members of the superfamily of heteropentameric ligand-gated ion channels that also include the nicotinic acetylcholine receptors, glycine receptors, and 5-HT3 receptors (Figure 1A) (reviewed in Unwin, 1989; Barnard et al., 1998). The subunits of all these receptors share a common ancestral structure that includes an extracellular N-terminal domain, four transmembrane domains (TM1-4), and an extended cytoplasmic loop region between TM3 and TM4 that mediates interactions with trafficking and signaling factors (Figures 1B and 1C). $GABA_A$R subunits are encoded by 19 different genes that have been

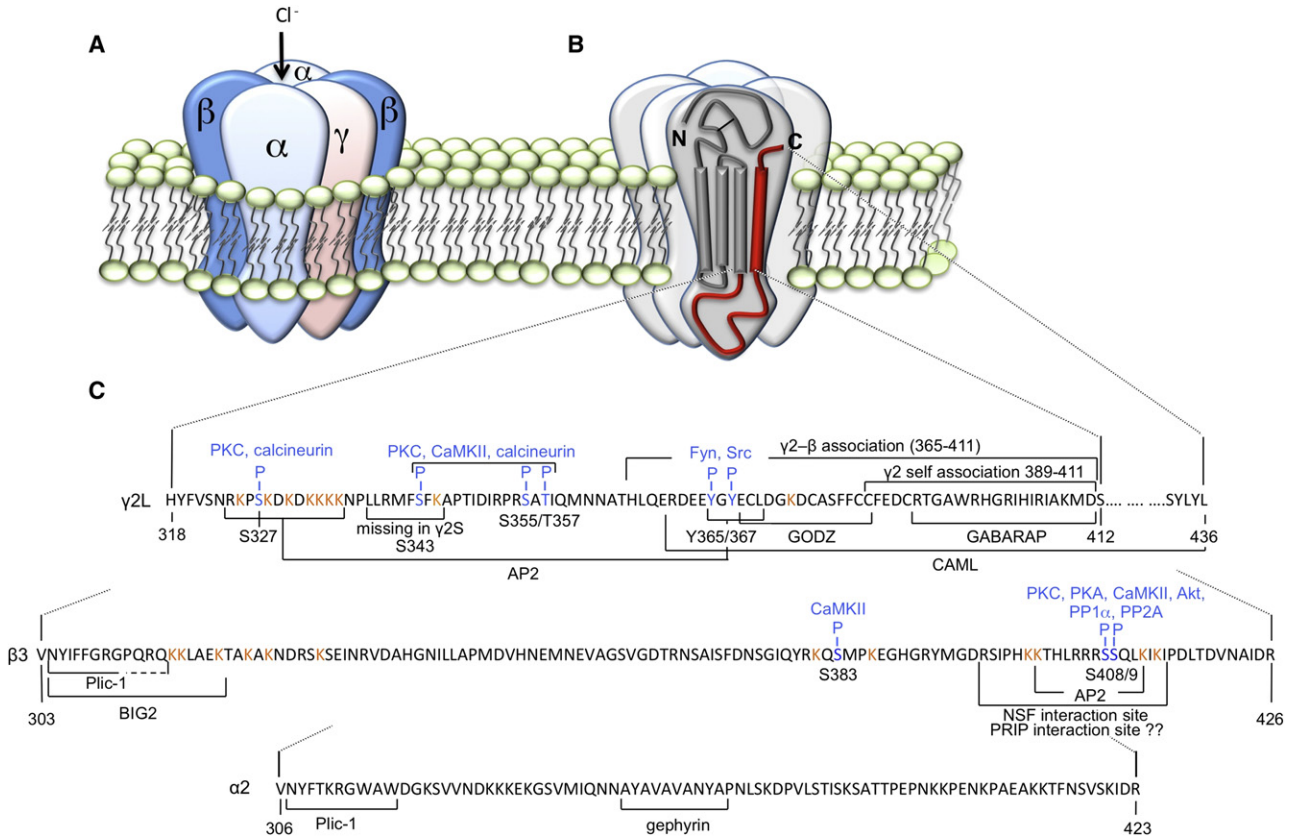

Figure 1. GABA$_A$R Subunit Structure and Intracellular Loop Sequences

(A) Schematic representation of GABA$_A$R heteropentamers consisting of two α, two β, and a single γ2 subunit assembled in a counterclockwise γ-β-α-β-α arrangement.

(B) Every subunit includes an extracellular N-terminal domain, four transmembrane domains (TM1-4) separated by an extended cytoplasmic loop region between TM3 and TM4, and a short extracellular C terminus. The cytoplasmic loop and TM4 regions of the γ2 subunit are essential for postsynaptic clustering of GABA$_A$Rs (Alldred et al., 2005).

(C) Sequences of cytoplasmic loop regions of representative subunits (γ2L, β3, α2) with amino acid numbers referring to mature polypeptides from the mouse. Interaction sites for binding partners are marked by brackets beneath the sequence, along with amino acid numbers of known Ser/Thr and Tyr phosphorylation sites. Phosphorylation sites are shown in blue; Lys residues representing putative ubiquitination sites are in orange. Note that the minimal interaction site for CAML includes the C-terminal region of the cytoplasmic loop as well as the TM4 domain of the γ2 subunit. For CaMKII phosphorylation sites see Houston et al. (2009). For other references see text.

grouped into eight subclasses based on sequence homology (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3). Alternative splicing contributes to additional receptor diversity. In particular, the γ2 (Whiting et al., 1990) and β2 subunits (McKinley et al., 1995) exist as short and long splice variants distinguished by the presence or absence of eight and 38 amino acids, respectively.

Different subunit combinations give rise to a large number of structurally and functionally distinct GABA$_A$R subtypes. Based on a recent conservative count, 11 structurally and functionally distinct receptor subtypes have been conclusively identified and are reasonably abundant in at least parts of the brain. They represent combinations of 2α and 2β subunits together with a single γ2 or δ subunit. An additional 15 receptor subtypes exist with high probability and a more limited distribution (Olsen and Sieghart, 2008). These numbers do not account for additional heterogeneity based on two different types of α or β subunits in one receptor complex (Khan et al., 1996; Benke et al., 2004), or due to alternative splicing of subunits. GABA$_A$Rs with different subunit compositions exhibit different pharmacology and channel gating properties, are differentially expressed during development and in the adult brain, accumulate at different neuronal cell surfaces, and are subject to differential regulation by extracellular cues.

The subsets of GABA$_A$Rs at synapses are composed of two α1, α2, or α3 subunits together with two β2 or β3 subunits and a single γ2 subunit. Compared to other GABA$_A$R subtypes discussed below, these receptors exhibit low affinity for GABA and thus are optimized to respond selectively to relatively high concentrations of GABA released into the synaptic cleft (300 μM, Perrais and Ropert, 1999). The γ2 subunit is essential for postsynaptic clustering of GABA$_A$Rs (Essrich et al., 1998). However, the γ3 subunit can substitute for the γ2 subunit and contribute to postsynaptic GABA$_A$Rs in the developing postnatal brain (Baer et al., 1999). Freeze-fracture replica immunogold labeling indicates that α2, α3, and β3 subunit-containing receptors are 50–130 times more concentrated at synapses than in the

extrasynaptic membrane (Kasugai et al., 2010). However, in absolute numbers the majority of these receptors are localized in the extrasynaptic space, which greatly exceeds the synaptic membrane area. Moreover, not all γ2-containing receptors are concentrated at synapses. In particular, α5βγ2 receptors are found almost exclusively at extrasynaptic sites (Brünig et al., 2002a; Crestani et al., 2002; Serwanski et al., 2006) and contribute to tonic GABAergic currents (Caraiscos et al., 2004; Glykys et al., 2008), although synaptic α5βγ2 receptors have been reported also (Serwanski et al., 2006; Zarnowska et al., 2009).

The most prominent population of nonsynaptic $GABA_A$Rs mediating tonic inhibition consists of α4βδ receptors in the forebrain and α6βδ receptors in the cerebellum. In addition, α1βδ receptors underlie tonic inhibition of hippocampal interneurons (Glykys et al., 2007). The δ-containing receptor subtypes exhibit high agonist affinity and therefore are tailored to function at ambient submicromolar concentrations of GABA outside of synapses (Saxena and Macdonald, 1996; Haas and Macdonald, 1999; Ke et al., 2000; Bianchi et al., 2001; Brown et al., 2002; Terpstra et al., 2002). Lastly, $GABA_A$Rs also are present on axons, including the axon initial segment of pyramidal cells (Nusser et al., 1996; Brünig et al., 2002a; Szabadics et al., 2006), mossy fiber terminals of hippocampal granule cells (Ruiz et al., 2003; Jang et al., 2006; Alle and Geiger, 2007), axon terminals of retinal bipolar neurons (Shields et al., 2000), and cerebellar parallel fibers (Stell et al., 2007). Axonal $GABA_A$Rs are thought to modulate action potential conductance and neurotransmitter release (Kullmann et al., 2005).

GABAergic Inhibition: Multiple Modes of Regulation

Changes in $GABA_A$R Subunit Gene Expression

Regulated expression of $GABA_A$R subunit genes determines cell type-specific and developmental changes in the subunit composition and function of $GABA_A$Rs. In addition, significant changes in subunit mRNA levels are observed in adulthood. For example, the subunit gene expression of α4βδ receptors in granule cells of the dentate gyrus is dynamically altered during epileptogenesis in a rat model of epilepsy (Brooks-Kayal et al., 1998; Peng et al., 2004) and during the estrus cycle of the mouse (Maguire et al., 2005). The levels of mRNAs encoding subunits of these receptors in CA1 pyramidal cells of rats is changed during puberty (Shen et al., 2007, 2010a), at the end of pregnancy (Sanna et al., 2009), and in a progesterone withdrawal model of premenstrual syndrome (Sundstrom-Poromaa et al., 2002). These studies in rodents indicate that alterations in subunit mRNA levels are generally paralleled by corresponding changes in the surface accumulation and function of $GABA_A$Rs that contribute to changes in neural excitability. Significant changes in subunit gene expression have also been described in postmortem brain of depressed patients and suicide victims (Klempan et al., 2009; Sequeira et al., 2009). Such changes are predicted to disrupt the subunit composition of $GABA_A$Rs and are consistent with the GABAergic deficit hypothesis of major depression (Luscher et al., 2011).

Dynamic Changes in the Cl^- Equilibrium Potential

The neural response to $GABA_A$R activation depends on the Cl^- equilibrium (E_{Cl}) potential, which determines the electrochemical driving force for Cl^-. E_{Cl} is determined chiefly by the relative expression of the Cl^- transporters KCC2 and NKCC1, which increase and decrease, respectively, during animal development and neural differentiation (for reviews see Ben-Ari, 2002; Fiumelli and Woodin, 2007; Andäng and Lendahl, 2008). The ensuing hyperpolarizing shift in E_{Cl} leads to a gradual conversion of GABAergic depolarization in immature neurons to mainly hyperpolarizing function in mature neurons. This switch in the function of $GABA_A$Rs is essential for structural and functional maturation of neurons (Tozuka et al., 2005; Ge et al., 2006; Cancedda et al., 2007) and for termination of interneuron migration in the developing neocortex (Bortone and Polleux, 2009). Recent evidence further suggests that the E_{Cl} of mature neurons may be subject to synaptic input-specific modulation by the voltage- and Cl^--sensitive Cl^- channel ClC-2 (Földy et al., 2010). The proposed function of ClC-2 is to prevent excessive accumulation of intracellular Cl^- following strong GABAergic stimulation. While GABAergic inputs to mature neurons are mostly inhibitory, depolarizing GABAergic effects are also common (reviewed by Marty and Llano, 2005; Kahle et al., 2008). In particular, the aforementioned axo-axonic synapses at the axon initial segment of cortical pyramidal cells (Szabadics et al., 2006), at hippocampal mossy fiber terminals (Jang et al., 2006), and on parallel fibers of the cerebellum (Stell et al., 2007; Pugh and Jahr, 2011) are depolarizing and excitatory due to the local absence of KCC2 (Szabadics et al., 2006). Moreover, dynamic changes in the functional expression of KCC2 can lead to pathophysiological adaptations of neural excitability. For example, chronic stress-induced downregulation of KCC2 results in a depolarizing shift of the chloride reversal potential of neurons in the paraventricular nucleus of the hypothalamus, which renders GABA inputs ineffective (Hewitt et al., 2009). This posttranslational mechanism is thought to contribute to hypothalamus-pituitary-adrenal (HPA) axis hyperactivity and to the neuropathology of stress-associated neuropsychiatric disorders. Moreover, KCC2 mRNA and/or protein expression is downregulated following focal ischemia (Jaenisch et al., 2010) and status epilepticus (Pathak et al., 2007). The sustained loss of GABAergic inhibition observed following status epilepticus has been proposed to underlie injury-induced long-term increases in seizure susceptibility. Mechanistically, status-epilepticus-induced downregulation of KCC2 involves BDNF/TrkB-mediated activation of cAMP response element-binding protein (CREB) (Rivera et al., 2004) and phosphorylation of tyrosine residues in the KCC2 C-terminal domain, which triggers its lysosomal degradation (Lee et al., 2010).

Regulation of $GABA_A$R Assembly

The functional expression of $GABA_A$Rs at the cell surface is first controlled at the level of assembly of subunits into heteropentameric complexes. A detailed understanding of this step is limited by the overabundance of different subunits coexpressed in individual neurons. Nevertheless, the use of concatenated subunit constructs representative of the most abundant $GABA_A$R subtype (α1β2γ2) established that assembly of heteropentamers follows strict rules, which ensure that the subunits assume a counterclockwise γ-β-α-β-α arrangement when viewed from

the synaptic cleft (Baumann et al., 2001, 2002; Baur et al., 2006). Interestingly, corresponding analyses of αβδ receptors indicate that the δ subunit does not simply take the place of the γ2 subunit. Instead the optimal subunit arrangement of δ-containing receptors depends on the type of α subunit present (Sigel et al., 2009). Forced expression of subunits in heterologous cells can lead to homomeric assemblies and complexes between α and γ or β and γ subunits that are, however, in most cases retained in the endoplasmic reticulum (ER) (Connolly et al., 1996). Formation of such nonproductive dimers or oligomers renders assembly of functional receptors rather inefficient, at least in heterologous cells (Gorrie et al., 1997). Unlike the α/γ or β/γ subunit combinations, coexpression of α and β subunits in heterologous cells results in formation of functional receptors that can reach the surface. Moreover, some evidence suggests that αβ receptors may exist naturally in small numbers and contribute to tonic inhibition of neurons (Brickley et al., 1999; Mortensen and Smart, 2006). However, when α, β, and γ2 subunits are coexpressed the formation of receptors containing all three types of subunits is strongly favored over receptors composed of α and β subunits alone (Angelotti and Macdonald, 1993). Moreover, single channel analyses of γ2 subunit knockout neurons indicate that receptors composed of α and β subunits alone are gated inefficiently by GABA and have much lower single channel conductances than naturally occurring receptors (Lorez et al., 2000).

The assembly of complexes that are translocated to the cell surface involves the initial formation of αβ subunit heterodimers and is principally controlled by the N-terminal/luminal domain of subunits (Taylor et al., 1999, 2000; Klausberger et al., 2000, 2001; Sarto et al., 2002; Bollan et al., 2003a; Ehya et al., 2003; Sarto-Jackson et al., 2006). This process involves interaction with ER-associated chaperones such as calnexin and binding immunoglobulin protein (BiP) (Connolly et al., 1996; Bradley et al., 2008). The fundamental role of α and β subunits in the assembly of $GABA_A$Rs is further evidenced by data from knockout mice, which indicate that deletion of α or β subunits results in loss of corresponding receptors (Homanics et al., 1997; Jones et al., 1997; Kralic et al., 2002a). By contrast, deletion of the γ2 subunit results in only a modest reduction of GABA binding sites (−22%), and the γ2 subunit is therefore largely dispensable for assembly of α and β subunits (Günther et al., 1995). Intriguingly, a recent study analyzing the expression of $GABA_A$Rs in transfected human embryo kidney (HEK) cells suggests that GABA might act as an intracellular chaperone important for $GABA_A$R biogenesis in the early secretory pathway (Eshaq et al., 2010). Consistent with such a function, the above-mentioned N-terminal assembly signals are located proximal to the GABA- and benzodiazepine-binding sites of $GABA_A$Rs (Boileau et al., 1999; Teissére and Czajkowski, 2001). The importance of subunit N-terminal domains for receptor assembly in vivo is exemplified by a naturally occurring point mutation (R43Q) in the γ2 subunit that is associated with childhood absence epilepsy and febrile seizures (Wallace et al., 2001; Kang and Macdonald, 2004; Hales et al., 2005; Frugier et al., 2007; Tan et al., 2007). Moreover, a small naturally occurring N-terminal deletion mutant of the rat α6 subunit abolishes assembly of corresponding receptors (Korpi et al., 1994).

The rules that govern differential assembly in cells that coexpress multiple $GABA_A$R subtypes remain little explored, although some evidence indicates that assembly may be mass-driven by the rate of cotranslation of compatible subunits. Transgenic mice that express ectopic α6 subunits in hippocampal pyramidal cells exhibit a gain of extrasynaptic α6βγ2 receptors at a cost of postsynaptic receptors (Wisden et al., 2002). Deletion of the α1 subunit in mice leads to compensatory upregulation of receptors containing other α subunits (Sur et al., 2001; Kralic et al., 2002a, 2002b, 2006). Furthermore, a residue (R66) in the N-terminal domain of the α1 subunit is essential for assembly of α1β2 receptors but dispensable for formation of α1β1 and α1β3 complexes (Bollan et al., 2003b). Recent evidence further suggests that entry of transport competent $GABA_A$R assemblies into the secretory pathway depends on subunit glycosylation (Tanaka et al., 2008; Lo et al., 2010).

Regulation of $GABA_A$R Exocytosis

The exit of $GABA_A$Rs from the ER

The exit of $GABA_A$Rs from the ER is limited by constitutive ER-associated degradation (ERAD) of α and β subunits (Gallagher et al., 2007; Saliba et al., 2007; Bradley et al., 2008), suggesting that receptor assembly is relatively inefficient (Figure 2). ERAD of $GABA_A$Rs is further enhanced by chronic blockade of neural activity (Saliba et al., 2009). Neural activity blockade-induced ubiquitination and degradation of $GABA_A$R subunits involves reduced Ca^{2+} entry through voltage-gated Ca^{2+} channels (VGCCs). The entry of $GABA_A$Rs into the secretory pathway is facilitated by interaction of α and β subunits with the *p*rotein that *l*inks *i*ntegrin-associated protein with the *c*ytoskeleton-1 (PLIC-1, also known as ubiquilin) (Bedford et al., 2001) (interaction sites of $GABA_A$R trafficking factors in $GABA_A$R subunit intracellular loop regions are indicated in Figure 1C). PLIC-1 is concentrated in the perinuclear ER in association with aggresomes (Heir et al., 2006) but also present in the nucleus (Mah et al., 2000) and in association with intracellular membranes in dendrites and near synapses (Bedford et al., 2001). PLIC-1 and its paralog PLIC-2 contain ubiquitin-like (ubl) proteasome binding domains and ubiquitin-associated (uba) domains, and the two proteins are known to interfere with ubiquitin-mediated proteolysis of diverse substrates (Wu et al., 1999; Kleijnen et al., 2000, 2003; Walters et al., 2002). Accordingly, overexpression of PLIC-1 in neurons promotes the surface expression of $GABA_A$Rs (Bedford et al., 2001), presumably by inhibiting ubiquitination and ERAD of α and β subunits (Figure 2).

ER to Golgi Translocation of $GABA_A$Rs

The γ2 subunit of $GABA_A$Rs is subject to palmitoylation at cytoplasmic cysteine residues, and this modification regulates the accumulation of $GABA_A$Rs at inhibitory synapses (Keller et al., 2004; Rathenberg et al., 2004). The Golgi-specific DHHC zinc finger protein (GODZ, zDHHC3) interacts with and palmitoylates the γ2 subunit in vitro (Figure 1C) (Keller et al., 2004; Fang et al., 2006). In brain, GODZ is selectively expressed in neurons and highly restricted to Golgi membranes, including Golgi outposts in primary dendrites (Keller et al., 2004). The protein is a member of a family of at least 23 structurally related palmitoyltransferases characterized by the presence of a DHHC motif-containing cysteine-rich domain (DHHC-CRD). Among these, only GODZ

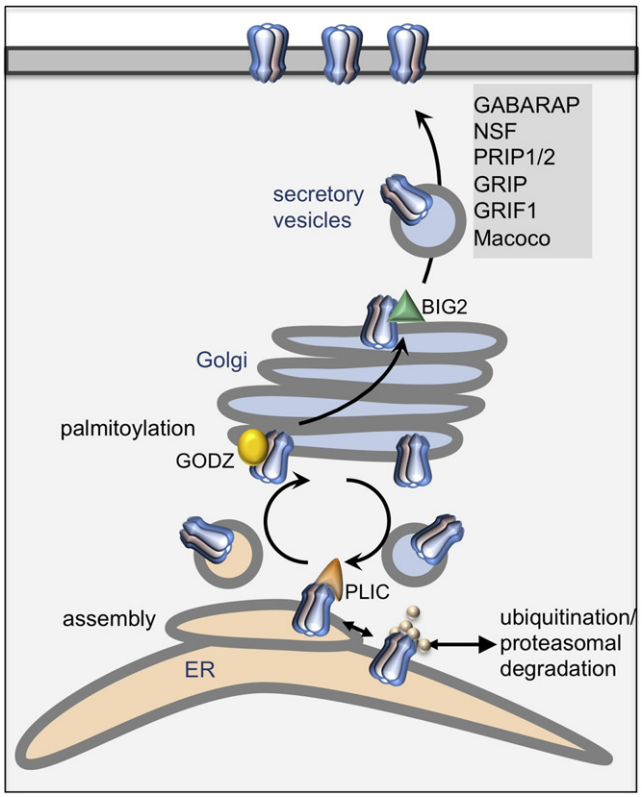

Figure 2. Regulated Trafficking of $GABA_AR$s in the Secretory Pathway

$GABA_AR$ heteropentamers assemble in the ER and interact with chaperones including calnexin and BiP. Unassembled or improperly folded receptor subunits are subject to ubiquitination and proteasomal degradation. This process is inhibited by interaction of α and β subunits with the ubiquitin-like protein PLIC, which in turn promotes the exit of receptors from the ER to the Golgi. The Golgi resident palmitoyltransferase GODZ palmitoylates the receptor γ2 subunit at cytoplasmic cysteine residues, which promotes translocation of receptors through the Golgi apparatus to the plasma membrane and to synapses. Exit of $GABA_AR$s from the Golgi may be facilitated by interaction of the GTP exchange factor BIG2 with $GABA_AR$ β subunits. The surface delivery of $GABA_AR$s is further promoted by a number of other proteins that currently cannot be assigned to a specific trafficking compartment (shaded gray).

and its paralog SERZ-β (zDHHC7) are able to palmitoylate the γ2 subunit in heterologous cells (Fang et al., 2006). Reducing the expression of GODZ by shRNA or dominant-negative constructs leads to selective loss of $GABA_AR$s at synapses, along with reduced GABAergic innervation and corresponding reductions in amplitude and frequency of miniature inhibitory synaptic currents (mIPSCs), as well as whole-cell currents (Fang et al., 2006). Palmitoylation is a reversible posttranslational modification and therefore may dynamically regulate the association of cytoplasmic substrates with membranous structures. In the case of integral membrane proteins, however, palmitoylation may extend the effective length of an adjacent transmembrane domain, as suggested by analysis of the palmitoylation-dependent trafficking of the Wnt coreceptor LRP6 (lipoprotein receptor-related protein 6) (Abrami et al., 2008). The restricted localization of GODZ to Golgi membranes, together with the notion that ER membranes are thinner than Golgi and plasma membranes (Bretscher and Munro, 1993; Mitra et al., 2004), suggests that GODZ serves to facilitate ER to Golgi translocation of γ2-containing $GABA_AR$s (Figure 2).

Translocation of $GABA_AR$s from Golgi to the Plasma Membrane

The brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2) interacts with a sequence motif in the intracellular loop of $GABA_AR$ β subunits that overlaps with the PLIC binding site (Figure 1C) (Charych et al., 2004b). BIG2 is a Sec7 domain-containing guanine exchange factor (GEF) that catalyzes GDP/GTP exchange of class I ADP-ribosylation factors (ARF) 1 and 3 (Morinaga et al., 1997; Togawa et al., 1999). GEF activation of these G-proteins is required for membrane budding of vesicles from the Golgi apparatus, thereby enabling proteins to proceed through the trans-Golgi network (TGN) toward the plasma membrane (Shin et al., 2004) (Figure 2). Coexpression of BIG2 with the β3 subunit in heterologous cells promotes the translocation of this subunit to the cell surface. Consistent with a role in exocytosis of $GABA_AR$s, BIG2 immunoreactivity is concentrated in the TGN and has been detected in somatic and dendritic vesicle-like structures, as well as in the postsynaptic density of both inhibitory and excitatory synapses (Charych et al., 2004b). Interestingly, independent studies have identified BIG2 as a component of recycling endosomes and provided evidence that BIG2-mediated activation of ARFs contributes to the structural integrity of this trafficking compartment (Shin et al., 2004, 2005; Boal and Stephens, 2010). Thus, BIG2 is implicated in facilitating the exit of $GABA_AR$s from the Golgi toward the plasma membrane as well as in endocytic recycling of $GABA_AR$s.

The $GABA_AR$ associated protein (GABARAP) represents the first $GABA_AR$ interacting protein isolated and accordingly has received considerable attention (Wang et al., 1999; reviewed in Chen and Olsen, 2007). It belongs to a family of ubiquitin-like proteins that in mammals includes the paralogs GEC-1 (guinea-pig endometrial cells-1, also known as GABARAP-like 1, GABARAPL1), GATE-16 (Golgi-associated ATPase enhancer of 16 kDa, also known as ganglioside expression factor-2 or GABARAPL2), GABARAPL3, GABARAPL4, and the more distantly related MAP-LC3 (microtubule-associated protein light chain 3). GABARAP interacts with all γ subunits and with microtubules in vitro and in vivo (Figure 1C) (Wang et al., 1999; Nymann-Andersen et al., 2002b). The protein is enriched in Golgi and other somatodendritic membrane compartments but absent at synapses (Kneussel et al., 2000; Kittler et al., 2001). Upon overexpression in hippocampal neurons, GABARAP facilitates the translocation of $GABA_AR$s to the cell surface (Leil et al., 2004). Interestingly, GABARAP-mediated trafficking of $GABA_AR$s involves an evolutionarily conserved lipid conjugation and delipidation cycle first described in yeast (Tanida et al., 2004). The attachment of phosphatidyl ethanolamine (PE) to the C terminus of GABARAP family proteins involves activating, conjugating, and deconjugating enzymes analogous to the ubiquitin conjugation system (Hemelaar et al., 2003; Tanida et al., 2003; Kabeya et al., 2004). Experiments in transfected cultured neurons indicate that conjugation to PE is required for dendritic accumulation of GABARAP and for GABARAP-induced cell surface expression of $GABA_AR$s (Chen and Olsen, 2007).

Activity-Induced Translocation of $GABA_A$Rs to the Plasma Membrane

Acute knockdown of GABARAP by siRNA in cultured neurons has revealed a role of GABARAP in rapid NMDA-induced functional plasticity of inhibitory synapses (Marsden et al., 2007). NMDA receptor-mediated Ca^{2+} influx following moderate stimulation of neurons with NMDA leads to a rapid increase in the number of postsynaptic $GABA_A$R clusters and mIPSC amplitudes (see also further below and Figure 5C). In addition to GABARAP this mechanism involves Ca^{2+} calmodulin-dependent kinase II (CaMKII), the vesicular trafficking factor N-ethylmaleimide-sensitive factor (NSF), and glutamate receptor interacting protein (GRIP). The rate of $GABA_A$R endocytosis following treatment with NMDA was unaltered, suggesting that GABARAP-dependent potentiation of inhibitory synapses involves increased exocytosis rather than reduced endocytosis of $GABA_A$Rs (Marsden et al., 2007). These findings represent thus far the only loss-of-function experiments showing an essential role for endogenous GABARAP in $GABA_A$R trafficking. The relevant protein-protein interactions and CaMKII phosphorylation targets have so far not been identified. However, experiments in heterologous cells allow speculation that this mechanism might involve CaMKII-induced phosphorylation of the β3 subunit at S383 (Houston et al., 2007).

GABARAP—A Multitasker?

The data summarized thus far suggest that GABARAP promotes the regulated, activity-dependent, and CaMKII-mediated translocation of $GABA_A$Rs from intracellular compartments to the somatodendritic plasma membrane. However, a more general role of GABARAP in exocytosis of $GABA_A$Rs is difficult to reconcile with other findings. First, GABARAP has been proposed to contribute to rebound potentiation, a neural activity-induced postsynaptic form of long-term potentiation (LTP) of inhibitory synapses on Purkinje cell neurons (Kawaguchi and Hirano, 2007). Using electrical stimulation of cultured Purkinje cells to mimic rebound potentiation, the authors found evidence that this form of plasticity is critically dependent on a CaMKII-dependent conformational alteration of GABARAP. However, LTP of GABAergic synapses occurred without measurable changes in the cellular distribution and cell surface expression of $GABA_A$Rs. Given that GABARAP is absent at synapses (Kneussel et al., 2000; Kittler et al., 2001), the exact role of GABARAP in this form of plasticity requires further clarification. Second, the aforementioned PE conjugation of GABARAP is critically involved in autophagy, an evolutionarily conserved form of bulk transport of membranes and cytoplasm to lysosomes for protein degradation (Tanida et al., 2004). Consistent with a role of GABARAP in autophagy, there is evidence that $GABA_A$Rs are subject to autophagy in worms. Body wall muscle cells of *C. elegans* lacking proper GABAergic and cholinergic innervation show selective accumulation of $GABA_A$Rs, but not nicotinic acetylcholine receptors, in autophagosomes. This indicates that in worms autophagy represents a pathway for endocytic lysosomal degradation of $GABA_A$Rs (Rowland et al., 2006). Third, GABARAP knockout mice show normal expression and punctate distribution of γ2-containing $GABA_A$Rs (O'Sullivan et al., 2005), possibly due to functional redundancy of GABARAP with GEC1 (Mansuy-Schlick et al., 2006) and other GABARAP family members. Fourth, the function of GABARAP is complicated by its interactions with a very large number of other proteins. Among these the ER luminal Ca^{2+}-dependent chaperone calreticulin stands out in that it binds GABARAP with exceptionally high affinity (Mohrlüder et al., 2007). Compared to calreticulin the interaction of GABARAP with γ2-derived peptides shows low affinity, suggesting that GABARAP might promote protein trafficking unspecifically along the secretory pathway (Knight et al., 2002).

Several GABARAP-interacting proteins contribute to $GABA_A$R trafficking independently of GABARAP. The aforementioned NMDAR-induced and GABARAP-dependent increase in $GABA_A$R clustering also depends on the synaptic PDZ domain-containing protein GRIP (Marsden et al., 2007), which interacts with GABARAP in vitro and in vivo (Kittler et al., 2004a). GRIP was first described as a trafficking factor of AMPARs (Dong et al., 1997). It is present at both glutamatergic and GABAergic synapses, consistent with functions at both types of synapses (Dong et al., 1999; Charych et al., 2004a; Kittler et al., 2004a; Li et al., 2005). GABARAP further interacts with the phospholipase C-related catalytically inactive proteins 1 and 2 (PRIP1/2, PRIP1 was previously named p130; Kanematsu et al., 2002), a pair of $GABA_A$R-associated adaptor proteins for phosphatases and kinases (Figure 3A) (Kanematsu et al., 2002; Uji et al., 2002). Likewise, GABARAP and its paralog GATE-16 (Sagiv et al., 2000) interact with NSF, an ATPase and chaperone of SNARE complexes that is critically important for regulated neurotransmitter release and also involved in trafficking of neurotransmitter receptors (Morgan and Burgoyne, 2004; Zhao et al., 2007).

Both PRIP1/2 and NSF interact with $GABA_A$Rs indirectly through GABARAP and directly via $GABA_A$R β subunits (Figure 1C) (Kanematsu et al., 2002; Kittler et al., 2004a; Terunuma et al., 2004; Goto et al., 2005). PRIP1/2 double knockout mice exhibit reduced expression and altered behavioral pharmacology of $GABA_A$Rs, suggesting deficits in mainly γ2-containing $GABA_A$Rs (Kanematsu et al., 2002, 2006; Mizokami et al., 2007). Brain extracts of these mice further show reduced association of $GABA_A$Rs with GABARAP, indicating that PRIP facilitates indirect association of GABARAP with $GABA_A$Rs (Mizokami et al., 2007). Moreover, PRIP and the γ2 subunit compete for binding to the same binding site on GABARAP (Kanematsu et al., 2002; Uji et al., 2002). The minimal interaction site for GABARAP in the γ2 subunit further overlaps with a γ2 domain that interacts with itself and with γ1, γ3, and β subunits in vitro (Figure 1C) (Nymann-Andersen et al., 2002a). Circumstantial evidence suggests that $GABA_A$Rs are endocytosed as dimers (Kittler et al., 2008). It is therefore conceivable that overexpressed GABARAP and γ2 subunit peptides designed to compete for interaction with GABARAP affect the surface expression of $GABA_A$Rs in part by competing for other protein interactions (receptor dimerization, interaction of receptors with PRIP and NSF) that facilitate the endocytic trafficking of $GABA_A$Rs.

Regulated Endocytosis of $GABA_A$Rs

Clathrin-Mediated Endocytosis

Regulated endocytosis of neurotransmitter receptors is known to underlie physiological and pathological adaptations of neural excitability. Endocytosis of $GABA_A$Rs occurs primarily

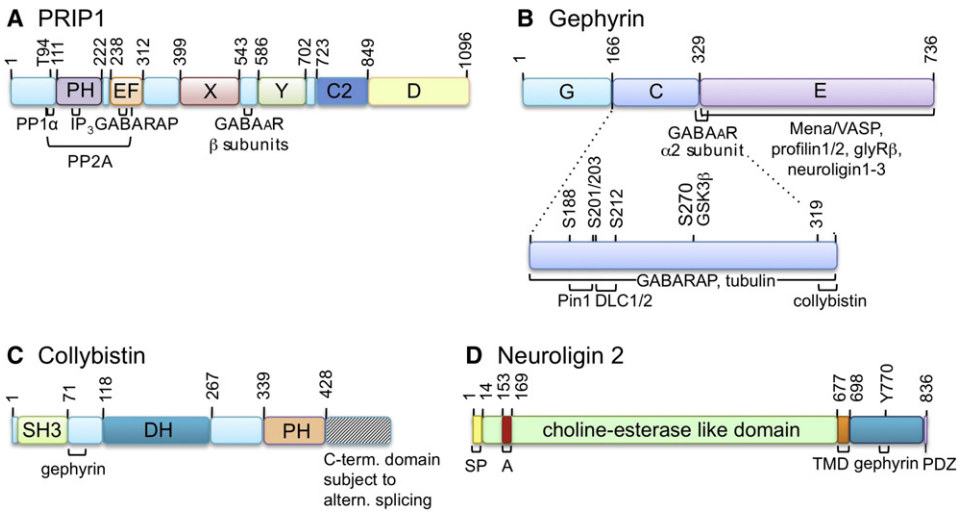

Figure 3. Schematic Representation of Proteins Pivotal for Intracellular Trafficking of Postsynaptic $GABA_AR$s

(A) PRIP consists of an N-terminal domain that incudes a binding site for the catalytic domain of PP1α, a PH domain that includes a binding site for IP3 (D-myo-inositol 1,4,5 triphosphate), an EF-hand domain that includes a binding site for GABARAP, and homologies to the catalytic (X, Y) and C2 domains of phospholipase Cδ. The $GABA_AR$ β subunit interaction domain is located between the X and Y domains of PRIP. Amino acid numbers refer to murine PRIP-1 (Kanematsu et al., 2005).

(B) Gephyrin consists of an N-terminal G-domain involved in the formation of gephyrin trimers, a central C domain with interaction sites for Pin1, DLC1/2, collybistin, a GSK3β phosphorylation site that regulates susceptibility to cleavage by calpain-1, and a C-terminal E domain that dimerizes in vitro and regulates clustering in vivo. In vitro assays suggest that the C domain also interacts with GABARAP and tubulin, although these proteins are not colocalized with postsynaptic gephyrin. The E domain binds Mena/VASP, profilin, the glycine receptor β subunit, and neuroligins. The gephyrin motif at the C-E domain interface that interacts with collybistin (PFPLTSMDKA) (Harvey et al., 2004) overlaps with the α2 subunit binding site (SMDKAFITVLEMPTVLGTE) (Saiepour et al., 2010). Amino acid numbers refer to rat gephyrin (Prior et al., 1992).

(C) Collybistin exists in three alternatively spliced versions that differ in sequence and length of their C-terminal domain (striped area). In addition, its clustering function is regulated by the presence or absence of an SH3 domain. Also shown are the dbl homology (DH) domain that regulates nucleotide exchange and the pleckstrin homology (PH) domain required for interaction with membrane phosphoinositides. The gephyrin binding site has been mapped to the linker domain between SH3 and DH domains. Amino acid numbers refer to rat collybistin (Kins et al., 2000).

(D) NL2 is composed of an N-terminal signal peptide (SP), followed by a large choline-esterase-like domain with the alternatively spliced exon A, a transmembrane domain (TM), an intracellular cytoplasmic domain that includes a 15 amino acid tyrosine-containing binding site for gephyrin, and a C-terminal binding site for PDZ domain proteins such as S-SCAM. Amino acid numbers refer to mouse NL2 (Ichtchenko et al., 1996).

via clathrin- and dynamin-dependent mechanisms that are facilitated by interactions of the $GABA_AR$ β and γ subunits with the clathrin adaptor protein AP2 (Kittler et al., 2000, 2005, 2008) (Figure 4). Accordingly, blocking the function of dynamin results in increased accumulation of postsynaptic $GABA_A$Rs along with increased mIPSC amplitudes (Kittler et al., 2000). When measured in about 1-week-old cultures, approximately 25% of cell surface $GABA_A$Rs are endocytosed within 30 min, and 70% of these receptors are recycled back to the cell surface within one hour. On a slower time scale (6 hr), about 30% of neuronal $GABA_A$Rs are targeted to late endosomes where they become subject to lysosomal degradation (Kittler et al., 2004b). However, constitutive endocytosis of $GABA_A$Rs is significantly reduced in mature neurons as indicated by studies that analyzed the diffusion dynamics of $GABA_A$Rs within the plasma membrane (discussed later in this review).

The search for sequence motifs important for AP2/clathrin/dynamin-mediated endocytosis of $GABA_A$Rs first led to the identification of a dileucine motif in β subunits that is critical for receptor internalization in heterologous cells (Herring et al., 2003, 2005). However, whether this particular mechanism operates in neurons has not been established. A second motif that is important for AP2/clathrin/dynamin-mediated $GABA_AR$ internalization in neurons has been mapped to a highly basic ten amino acid sequence motif that includes a major phosphorylation site conserved in the cytoplasmic loop region of β1-3 subunits (S408, S409 in β3, Figure 1C) (Kittler et al., 2005, 2008). Importantly, interaction of the AP2 μ2 subunit with this site is negatively regulated by phosphorylation of $GABA_AR$ β subunits, indicating that AP2 binds $GABA_A$Rs with high affinity and triggers their internalization preferentially when this site is dephosphorylated. Accordingly, perfusion of neurons with an unphosphorylated β3-derived peptide that competes with this interaction results in enhanced mIPSCs and whole-cell currents (Kittler et al., 2005).

Regulation of Endocytosis by Phospho-sensitive Interactions with $GABA_AR$ β Subunits

The AP2 interaction site on β1 and β3 subunits (Figure 1C) can be phosphorylated by both protein kinase A (PKA) (McDonald et al., 1998) and protein kinase C (PKC) (Brandon et al., 2000; Brandon et al., 2002), while the same site in the β2 subunit is phosphorylated by PKC only (McDonald et al., 1998; Brandon et al., 2003), allowing for receptor subtype-specific modulation of $GABA_AR$ endocytosis. However, the same site can also be phosphorylated by CaMKII (McDonald and Moss, 1994) and Akt (also known as PKB) (Wang et al., 2003b; Xu et al., 2006). The latter is discussed further below in the context of insulin-induced exocytosis of $GABA_A$Rs. PKC-mediated phosphorylation is

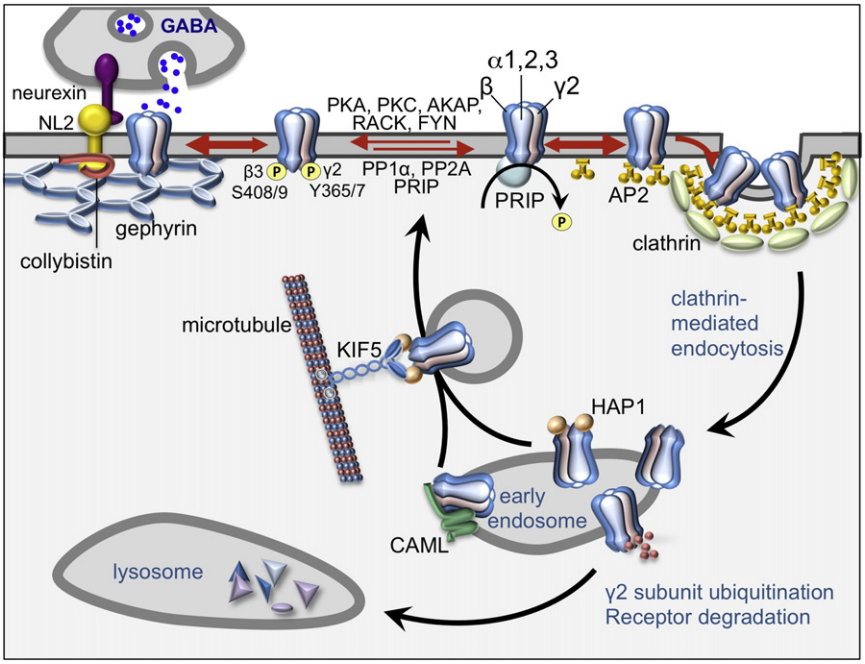

Figure 4. Regulated Endocytosis and Recycling of $GABA_AR$s

$GABA_AR$ endocytosis through clathrin-coated vesicles is regulated by phospho-sensitive interactions of β and γ2 subunits with the clathrin adaptor AP2. Phosphorylation of β subunits (S408/409 in β3) and the γ2 subunit (Y365/367) by PKA/PKC and Fyn/Src, respectively, interferes with these interactions and thereby stabilizes $GABA_AR$s at the cell surface. Phosphorylation of β subunits by PKA and PKC is facilitated by the kinase adaptors AKAP and RACK, respectively. Dephosphorylation is modulated by PRIP-associated PP1α and PP2A. $GABA_AR$s in the plasma membrane are subject to lateral diffusion. Interaction of $GABA_AR$s with gephyrin (i.e., through α2/3 subunits) and collybistin (α2 subunit) leads to their accumulation at synapses. Interaction of gephyrin/collybistin/$GABA_AR$ complexes with the NL2-neurexin synaptic adhesion complex contributes to proper alignment of pre- and postsynaptic complexes at inhibitory synapses. Conversely, dephosphorylation of β subunits by PP1α and PP2A (β3 subunit S408/409 site) and unidentified tyrosine phosphatases (γ2 Y365/367) facilitates interaction of extrasynaptic $GABA_AR$s with AP2, which then triggers clathrin-mediated internalization. Dephosphorylation of β subunits by PP1α is inhibited or facilitated by the phosphatase adaptor PRIP, depending on its own phosphorylation state. Endocytosed receptors in early endosomes are ubiquitinated at lysine and possibly other residues of the γ2 subunit, which then leads to lysosomal degradation. Alternatively, interactions of CAML with the γ2 subunit cytoplasmic and transmembrane domains and of HAP1 with the β subunit cytoplasmic domain facilitate KIF5-dependent vesicular transport and recycling of $GABA_AR$s to the plasma membrane.

facilitated by stable interaction of this kinase with β subunits, either directly as shown for the PKC-βII isozyme or indirectly through the receptor for activated C-Kinase (RACK-1), which recognizes a binding site in the β1 subunit adjacent to the PKC binding site (Brandon et al., 1999, 2002). Reductions in the PKC-mediated phosphorylation of $GABA_AR$ β subunits are implicated in the dramatic loss of GABAergic inhibition in animal models of status epilepticus, which is thought to underlie pharmaco-resistance to benzodiazepines following prolonged seizures in epileptic patients (Terunuma et al., 2008).

The β subunit phosphostate-dependent endocytosis of $GABA_AR$s is further regulated by interaction of β subunits with PRIP1/2 and their function as adaptors for the serine/threonine-specific phosphatases PP1α and PP2A (Yoshimura et al., 2001; Uji et al., 2002; Terunuma et al., 2004; Kanematsu et al., 2006, 2007). Phosphorylation of PRIP at a threonine residue (T94 in PRIP1) leads to dissociation of the catalytically inactive PRIP/PP1α complex and activation of PP1α and hence dephosphorylation of the β3 subunit at the AP2 interaction site (Terunuma et al., 2004). Unlike PP1α, PP2A is constitutively active when bound to PRIP (Kanematsu et al., 2006). Consistent with a role of PRIP-associated phosphatases in endocytosis of $GABA_AR$s, the PRIP/PP1α/PP2A complex can be coimmunoprecipitated with AP2 and clathrin from brain extracts (Kanematsu et al., 2007). Moreover, PRIP facilitates $GABA_AR$ endocytosis in transfected heterologous cells. The association of PRIP with PP2A (Kanematsu et al., 2006) is implicated in brain-derived neurotrophic factor (BDNF)-induced downregulation of $GABA_AR$s (Jovanovic et al., 2004), as discussed in further detail below. The end effect of PRIP on $GABA_AR$ cell surface expression appears to depend on the cellular state of several other signal transduction pathways. The aforementioned phenotype of PRIP1/2 double knockout mice, which includes functional deficits of $GABA_AR$s, suggests that PRIP primarily facilitates the exocytosis or cell surface stability of $GABA_AR$s (Kanematsu et al., 2002, 2006; Mizokami et al., 2007). Recent evidence summarized further below indicates that PRIP1/2 also serve as an adaptor for the serine/threonine kinase Akt, which promotes the de novo insertion of $GABA_AR$s into the plasma membrane (Fujii et al., 2010).

Intriguingly, the AP2 interaction site in the β1-3 subunits overlaps with the binding site for the vesicular ATPase and trafficking factor NSF (Figure 1C) (Goto et al., 2005). NSF interacts with phorbol ester-activated PKCε. Moreover, PKCε phosphorylates and activates the ATPase function of NSF. PKCε-mediated phosphorylation of NSF induces its translocation to the plasma membrane and to synapses and concurrently reduces the cell surface expression of $GABA_AR$s (Chou et al., 2010). PKCε knockout mice are less anxious and produce lower levels of stress hormone than WT mice (Hodge et al., 2002), which is the opposite of the anxious-depressive-like phenotype of $GABA_AR$ γ2 subunit heterozygous mice and therefore consistent with increased functional expression of $GABA_AR$s (Crestani et al., 1999; Luscher et al., 2011). Therefore, pharmacological inhibitors of PKCε activity may have therapeutic potential for the treatment of neuropathological conditions that involve deficits in GABAergic transmission. This NSF-dependent trafficking mechanism is reminiscent of aforementioned earlier experiments conducted in heterologous cells, showing phorbol ester-induced and PKC and clathrin-mediated endocytosis of

GABA$_A$Rs from the plasma membrane by a mechanism that is independent of GABA$_A$R phosphorylation (Chapell et al., 1998; Connolly et al., 1999). PKCε is one of seven PKC isozymes activated by phorbol esters. It therefore seems likely that PKCε contributes to phorbol ester-induced endocytosis of GABA$_A$Rs. However, one might predict that PKCε and NSF-dependent endocytosis of GABA$_A$Rs is counteracted by the aforementioned PKC-βII-mediated phosphorylation of β subunits, which limits endocytosis of GABA$_A$Rs. Consistent with multiple PKC and PKA-regulated modes of GABA$_A$R trafficking, these kinases can have cell-type-specific and functionally opposite effects on mIPSC amplitudes in vivo (Poisbeau et al., 1999).

Regulation of Endocytosis by Phospho-sensitive Interactions with the GABA$_A$R γ2 Subunit

A third interaction of GABA$_A$Rs with AP2 involves a bipartite motif in the intracellular loop region of the γ2 subunit (Figure 1C). It consists of a 12 amino acid basic domain that is homologous to the AP2 binding site in β subunits and a more C-terminal γ2-specific YGYECL motif (Smith et al., 2008). These two domains interact cooperatively with separate domains in the μ2 subunit of AP2. The γ2-specific YGYECL motif is of particular interest as it exhibits high affinity for AP2 that is sensitive to phosphorylation at γ2 Tyr365/367 (Kittler et al., 2008). These residues are phosphorylated by Fyn and other Src kinase family members in vivo (Lu et al., 1999; Jurd et al., 2010). A non-phosphorylated YGYECL peptide effectively competes with the AP2-γ2 subunit interaction, thereby increasing the GABA$_A$R surface expression and mIPSC amplitude and showing that this site is constitutively phosphorylated in cultured neurons (Kittler et al., 2008). This mechanism is also important in vivo as evidenced by reduced expression and altered function of GABA$_A$Rs in Fyn knockout mice (Boehm et al., 2004) and by an embryonic lethal phenotype of knockin mice in which the γ2 Tyr365/367 residues were mutated to phenylalanine, which interferes with AP2 binding (Tretter et al., 2009). Heterozygous $\gamma2^{Y365/7F}$ mice, however, are viable. In the stratum pyramidale of the hippocampus they show a CA3-region-specific increase in the postsynaptic accumulation of GABA$_A$Rs, suggesting different basal levels of γ2 Tyr365/367 phosphorylation in the CA3 versus CA1 region. The lethal phenotype of homozygous $\gamma2^{Y365/7F}$ mutants indicates that excessive GABAergic transmission is detrimental during early development, probably due to excessive GABAergic excitation, which may interfere with normal neurogenesis and neural migration (Wang and Kriegstein, 2009).

Collectively, there is now conclusive evidence that GABA$_A$Rs are subject to at least two major mechanisms of regulated endocytosis. These mechanisms involve different phospho-sensitive interactions of the clathrin adaptor AP2 with β and γ2 subunits, respectively. The phospho-states of the relevant β and γ2 subunit motifs are subject to regulation by multiple Ser/Thr and Tyr kinases, as well as phosphatases and their respective adaptor proteins. Dynamic changes in the phosphorylation state of NSF and PRIP and their interaction with the AP2 binding site of β subunits provide additional levels of regulation. Future experiments will need to address whether NSF and PRIP compete with AP2 for GABA$_A$R interaction and whether their interaction with GABA$_A$Rs is regulated by phosphorylation of GABA$_A$Rs.

Regulation of Recycling and Degradation of GABA$_A$Rs

The decision of whether endocytosed GABA$_A$Rs are recycled or degraded is regulated by interaction of GABA$_A$R β1-3 subunits with huntingtin-associated protein 1 (HAP1) (Figure 4) (Kittler et al., 2004b). HAP1 interacts with the Huntington disease protein huntingtin (Li et al., 1995; Li et al., 2002) and is involved in motor-protein-dependent transport of neuronal cargo (Engelender et al., 1997; Gauthier et al., 2004; McGuire et al., 2006). When overexpressed in cultured neurons, HAP1 interferes with the degradation of endocytosed GABA$_A$Rs and thereby increases the recycling and surface expression of GABA$_A$Rs (Kittler et al., 2004b). More recent experiments have identified HAP1 as an adaptor for the kinesin superfamily motor protein 5 (KIF5), interacting directly with all three isoforms (A-C) of KIF5 heavy chains (Twelvetrees et al., 2010). HAP1, KIF5 heavy chains and γ2-containing GABA$_A$Rs are partly colocalized in dendrites and can be isolated as a complex from brain lysates. Moreover, live imaging and electrophysiological recordings revealed that HAP1-KIF5-dependent vesicular trafficking controls the delivery of GABA$_A$Rs to the plasma membrane and thereby promotes the function of GABAergic inhibitory synapses. Interestingly, mutant huntingtin with an expanded polyGln repeat that causes Huntington's disease interferes with normal HAP1-KIF-dependent vesicular transport and thereby reduces the amplitude of GABAergic mIPSCs. Thus, reduced expression and function of GABA$_A$Rs may contribute to neurodegeneration associated with Huntington's disease (Twelvetrees et al., 2010).

Of note, the γ-aminobutyric acid(A) receptor-interacting factor, GRIF-1 (also known as TRAK2, OIP98, ALS2CR3, huMilt2), which has been shown to interact selectively with the β2 subunit in vitro (Beck et al., 2002), also interacts with KIF5 motor proteins (Brickley et al., 2005). The precise function of GRIF-1 in trafficking of GABA$_A$Rs is unknown but the protein provides a second potential link between GABA$_A$Rs and the KIF5 vesicular trafficking machinery. Furthermore, the GRIF-1 paralog TRAK1, which also interacts with KIF5 (Brickley et al., 2005), has been isolated as the gene that causes a spontaneous hypertonic mutant phenotype of mice associated with elevated basal activity of motor neurons (Gilbert et al., 2006). TRAK1 can be immunoprecipitated with GABA$_A$Rs from brain extracts and results in reduced GABA$_A$R immunostaining when mutated, probably due to a dominant-negative effect of mutant TRAK1. Consistent with an underlying GABA$_A$R deficit, the hypertonic phenotype of TRAK1 mutants can be ameliorated by potentiation of GABA$_A$R function with benzodiazepines (Gilbert et al., 2006).

An independent line of experiments identified calcium-modulating cyclophilin ligand (CAML) as a regulator of postendocytic trafficking of GABA$_A$Rs (Figure 4) (Yuan et al., 2008). CAML is an integral membrane protein that is essential for normal embryonic development and for differentiation of neurons in culture. However, conditional deletion of CAML in differentiated neurons results in reduced accumulation of GABA$_A$Rs at the plasma membrane and at synapses, along with selective GABAergic but not glutamatergic functional deficits. Interestingly, CAML interacts with the C-terminal cytoplasmic and transmembrane

domains of γ subunits (Yuan et al., 2008). These domains are essential for clustering and function of $GABA_A$Rs at synapses, as was shown for the γ2 subunit (Alldred et al., 2005; Christie et al., 2006). Reduced plasma membrane accumulation and function of $GABA_A$Rs in CAML-deficient neurons is associated with normal endocytosis from the plasma membrane but reduced recycling of $GABA_A$Rs from endocytic pools (Yuan et al., 2008). This function of CAML in endocytic recycling of $GABA_A$Rs is consistent with a similar role of CAML in recycling of endocytosed epidermal growth factor (EGF) receptor (Tran et al., 2003).

A recent report has identified Maf1 and a Maf1-interacting coiled-coil protein named Macoco as additional $GABA_A$R β3 subunit interacting proteins (Smith et al., 2010). Maf1 was originally identified in yeast as a nuclear regulator of t-RNA transcription (Pluta et al., 2001). However, in neurons Maf1 is also present in the somatodendritic cytoplasm. Macoco was isolated as a Maf1-interacting protein and then found to also interact with $GABA_A$R γ2 and β3 subunits independently of Maf1. Both proteins are highly expressed in hippocampus, and they are partially colocalized with postsynaptic $GABA_A$Rs in cultured neurons. Overexpression of Macoco facilitates the surface expression of $GABA_A$Rs, suggesting a function in the secretory pathway (Smith et al., 2010). However, the precise mechanism for this effect remains to be determined.

Endocytosed $GABA_A$Rs that fail to be recycled are targeted for lysosomal degradation as demonstrated by reduced degradation in the presence of the lysosomal protease inhibitor leupeptin (Figure 4) (Kittler et al., 2004b). This route of trafficking is facilitated by ubiquitination of a series of lysine residues within the intracellular domain of the γ2 subunit (Figure 1C). Blockade of lysosomal activity or disruption of the trafficking of ubiquitinated cargo to lysosomes specifically increases the accumulation of $GABA_A$Rs at synapses as well as the efficacy of GABAergic synaptic inhibition (Arancibia-Cárcamo et al., 2009). Moreover, mutation of the cytoplasmic γ2 Lys residues retards the lysosomal targeting of $GABA_A$Rs and is sufficient to block the loss of synaptic $GABA_A$Rs induced by anoxic insult. Thus, in addition to ubiquitin-mediated proteasomal degradation of α and β subunits at the ER, the number of $GABA_A$Rs at synapses is also regulated by ubiquitin-mediated degradation of the γ2 subunit in the endocytic lysosomal pathway (Arancibia-Cárcamo et al., 2009). The ubiquitin ligases involved in degradation of $GABA_A$Rs are not yet known. However, a recent preliminary report has identified brain-expressed ring finger protein (BERP, also known as TRIM3, RNF22) as a putative ubiquitin ligase that, counterintuitively, facilitates the cell surface expression and synaptic function of $GABA_A$Rs (Cheung et al., 2010). Whether BERP acts directly on $GABA_A$Rs or other protein(s) as a substrate has not yet been determined.

The mechanisms of endocytic recycling summarized above have been explored with a focus on γ2-containing $GABA_A$Rs that are confined to synapses. Emerging evidence indicates that similar mechanism may apply to nonsynaptic, δ-containing receptors. In particular, phosphorylation of Ser443 in the α4 subunit promotes the cell surface stability of α4βδ receptors (Abramian et al., 2010).

Regulation of $GABA_A$R Interactions with the Postsynaptic Protein Scaffold

Molecular imaging of bungarotoxin-labeled recombinant $GABA_A$Rs suggests that the delivery to the cell surface and endocytosis occur at nonsynaptic plasma membrane sites (Bogdanov et al., 2006). Consistent with these observations, the insertion of $GABA_A$Rs into the plasma membrane can proceed normally in the absence of subsynaptic scaffold proteins (Lévi et al., 2002; Lévi et al., 2004). However, the distribution of $GABA_A$Rs between synaptic and extrasynaptic sites in the plasma membrane is dynamically regulated by direct and indirect interactions of $GABA_A$Rs with the postsynaptic scaffold, as detailed in the following.

Gephyrin

Arguably the most important protein for stabilization of $GABA_A$Rs at synapses is gephyrin, the principal subsynaptic scaffold protein of both GABAergic and glycinergic synapses (Figures 3B and 5A) (Fritschy et al., 2008). Gephyrin was first identified as a 93 KDa polypeptide that copurified with affinity-purified glycine receptors (Pfeiffer et al., 1982), the principal inhibitory neurotransmitter receptors in the spinal cord. Molecular cloning and targeted deletion in mice revealed that gephyrin is a multifunctional protein that is broadly expressed and essential for postsynaptic clustering of glycine receptors and also for molybdenum cofactor (Moco) biosynthesis in nonneural tissues (Prior et al., 1992; Kirsch et al., 1993; Feng et al., 1998; Sola et al., 2004; Dumoulin et al., 2009). Gephyrin interacts with microtubules (Kirsch et al., 1995) as well as several regulators of microfilament dynamics including profilin I and II (Mammoto et al., 1998) and members of the mammalian enabled (Mena)/vasodilator-stimulated phosphoprotein (VASP) family (Figures 3B and 5A) (Giesemann et al., 2003). The N-terminal gephyrin domain known as G-gephyrin assumes a trimeric structure (Schwarz et al., 2001; Sola et al., 2001), whereas the C-terminal E domain forms a dimer (Schwarz et al., 2001; Xiang et al., 2001; Sola et al., 2004). These domain interactions are essential for oligomerization and clustering of gephyrin at postsynaptic sites (Saiyed et al., 2007). The clustering function of gephyrin is regulated by select residues within the E-domain that are dispensable for E-domain dimerization (Lardi-Studler et al., 2007). Moreover, the linker region between E and G domains of gephyrin is thought to interact with microtubules (Ramming et al., 2000). Thus, gephyrin has the structural prerequisites to form a microtubule and microfilament-associated hexagonal protein lattice that may organize the spatial distribution of receptors and other proteins in the postsynaptic membrane.

Gephyrin has long been established as a phosphoprotein (Langosch et al., 1992), although to date few studies have addressed the relevance of this modification. Zita et al. (2007) showed preliminary evidence that gephyrin is phosphorylated by proline-directed kinase(s) and that this is essential for interaction of gephyrin with the peptidyl-prolyl *cis/trans* isomerase Pin1 (Figure 5A). Pin1-induced conformational changes of gephyrin were found to be essential for maximal clustering of glycine receptors, suggesting a similar function for Pin1 in regulating gephyrin destined for GABAergic synapses. Recently, an unbiased proteomic screen using mass spectrometry mapped the first specific phosphorylation sites to S188, S194, and

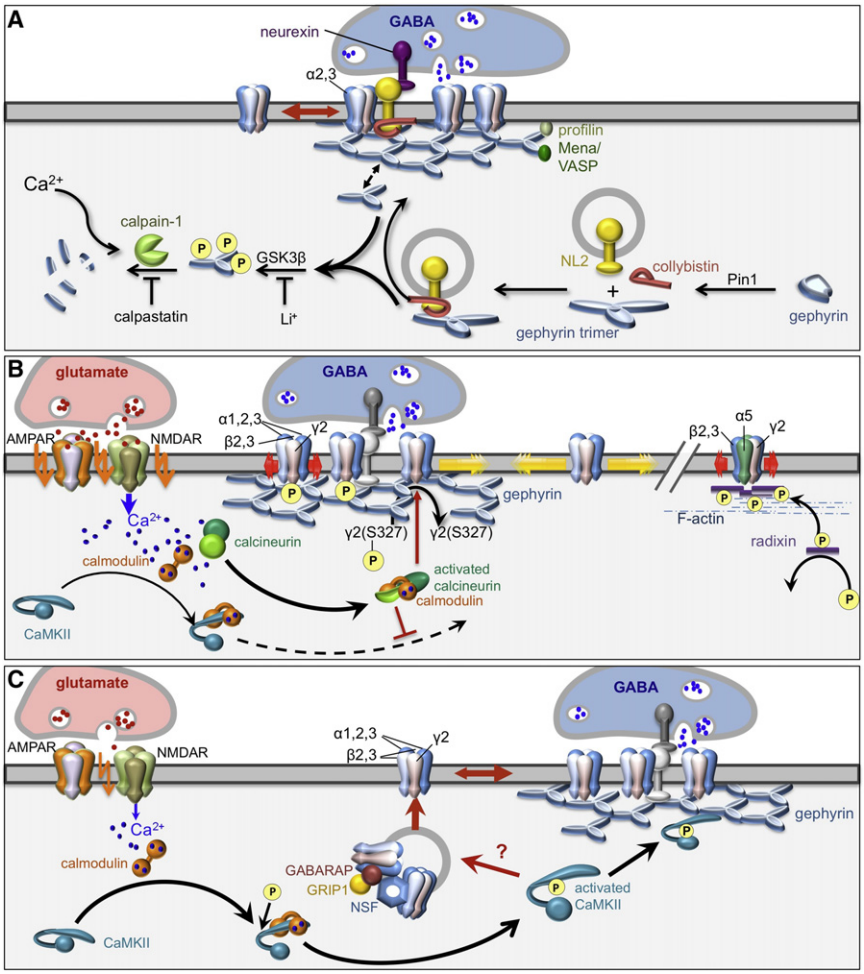

Figure 5. Regulation of $GABA_AR$ Clustering and Lateral Mobility at Synaptic and Extrasynaptic Sites

(A) The biosynthesis of gephyrin is regulated by the peptidyl-prolyl *cis/trans* isomerase Pin1. Cytosolic soluble gephyrin exists as a trimer. The deposition of gephyrin trimers at the plasma membrane is facilitated by cooperative interactions of gephyrin with CB^{SH3+} (tethered to the plasma membrane by phosphoinositide binding of its PH domain) and NL2, which unlock the CB^{SH3+}-dependent clustering function, presumably by releasing an intramolecular inhibition of CB^{SH3+} by its SH3 domain. The gephyrin/NL2/collybistin complex enables the postsynaptic clustering of gephyrin and, through interaction with presynaptic neurexins, helps to align the postsynaptic complex with GABAergic terminals. The $GABA_AR$ α2 subunit may substitute for NL2 and enable collybistin-dependent clustering of gephyrin. The clustering of $GABA_ARs$ in the postsynaptic specialization is facilitated by interaction of specific subunits (α2, α3) with gephyrin. Postsynaptic gephyrin further interacts with Mena/VASP and profilin I/II. Competition of gephyrin and G-actin for interaction with profilin I/II is implicated in regulation of the microfilament-dependent receptor packing density. The density of postsynaptic gephyrin clusters is regulated by GSK3β-mediated phosphorylation of gephyrin, which enhances the susceptibility of gephyrin to cleavage by the Ca^{2+}-dependent protease calpain-1. Constitutive proteolytic cleavage of gephyrin limits the confinement and accumulation of postsynaptic $GABA_ARs$, by facilitating their lateral diffusion. Conversely, inhibition of GSK3β by Li^+ or of calpain-1 by its natural antagonist calpastatin stabilizes gephyrin and thereby promotes the density of gephyrin clusters as well as GABAergic synaptic function.

(B) Postsynaptic $GABA_ARs$ (α1,2,3β2/3γ2) are confined (red arrows) by interactions with gephyrin and presumably other postsynaptic scaffold proteins. However, on leaving this area they become highly mobile within the plane of the phospholipid bilayer (yellow arrows). The interaction of $GABA_ARs$ with the postsynaptic cytoskeleton is regulated by the activity-dependent and calcineurin-regulated phosphorylation state of the γ2 subunit. Robust excitation of glutamate receptors leads to NMDAR/Ca^{2+}- and Ca^{2+}/calmodulin-mediated activation of calcineurin and dephosphorylation of γ2(S270), which reduces the postsynaptic confinement of $GABA_ARs$, allowing their diffusion away from synapses. CaMKII is activated in parallel but its translocation to synapses is prevented by calcineurin by an unknown mechanism. In contrast to α1,2,3β2/3γ2 receptors, α5βγ2 receptors are clustered extrasynaptically by interaction with phospho-activated radixin, which links these receptors to submembrane microfilaments.

(C) Modest stimulation of neurons as mimicked by treatment of neurons with NMDA leads to more limited influx of Ca^{2+} and preferential activation of CaMKII. Activated CaMKII is translocated to synapses and stimulates the insertion of $GABA_ARs$ into the plasma membrane where they are trapped at synapses by interaction with the postsynaptic cytoskeleton. Insertion of $GABA_ARs$ into the plasma membrane involves GABARAP, NSF, and GRIP. The relevant target proteins interacting with and phosphorylated by CaMKII are not yet known.

S200 of gephyrin (Huttlin et al., 2010). Treatment of cultured neurons with inhibitors of the phosphatases PP1α and PP2A caused a significant loss of gephyrin from inhibitory synapses (Bausen et al., 2010). However, mutation of S188/194 to alanine or glutamate resulted in only a modest change in gephyrin or $GABA_AR$ cluster size, indicating that the effect of phosphatases was due to dephosphorylation of other PP1α/PP2A substrates.

An elegant study has identified glycogen synthase kinase 3β (GSK3β) as a proline-directed kinase that controls phosphorylation- and proteolytic cleavage-induced turnover of gephyrin (Figure 5A) (Tyagarajan et al., 2011). Using tandem mass spectrometry of gephyrin, the authors identified S270 as a residue that is basally phosphorylated in brain tissue. Transfection of cultured neurons with phosphorylation-deficient $gephyrin^{S270A}$ increased the density of gephyrin clusters and the amplitude and frequency of GABAergic mIPSCs, indicating that gephyrin clustering is limited by phosphorylation at S270. However, mutations of S270 had no effect on cluster size. Using kinase-specific inhibitors in in vitro phosphorylation assays the authors identified GSK3β as an important kinase for S270. To address the mechanism by which phosphorylation might increase gephyrin turnover they focused on calpain-1. This Ca^{2+}-dependent cysteine protease was previously shown to cleave gephyrin and to produce a stable C-terminal gephyrin fragment of 48–50 kDa (Kawasaki et al., 1997). Transfection of neurons with the natural calpain-1 inhibitor calpastatin increased the gephyrin cluster density (Tyagarajan et al., 2011). Moreover, this effect was enhanced in the presence of the phosphomimetic mutant $gephyrin^{S270E}$ as a substrate, indicating that calpain-1-mediated

degradation of gephyrin is triggered by phosphorylation of S270. Lastly, the authors showed that S270 phosphostate-dependent clustering of gephyrin is enhanced by chronic treatment of cultured neurons or mice with Li^+, a potent inhibitor of GSK3β used as mood-stabilizing agent for the treatment of bipolar disorder. The findings strongly suggest that Li^+-induced enhancement of GABAergic synaptic transmission contributes to the mood-stabilizing effects of Li^+ in patients (Tyagarajan and Fritschy, 2010). GSK3β is inhibited as a downstream target of both the canonical Wnt signaling pathway (Inestrosa and Arenas, 2010) and the insulin receptor signaling pathway. Both pathways promote the postsynaptic clustering of $GABA_A$Rs by additional, gephyrin-independent mechanisms, as detailed further below.

Gephyrin forms a stable complex with affinity-purified glycine receptors (Pfeiffer et al., 1982). By contrast, $GABA_A$Rs in detergent-solubilized membrane extracts do not stably associate with gephyrin (Meyer et al., 1995). Moreover, a major subset of $GABA_A$Rs comprising α1βγ2 receptors can accumulate and cluster at synapses independently of gephyrin (Kneussel et al., 2001; Lévi et al., 2004). Nevertheless, in brain gephyrin serves as a reliable postsynaptic marker for all GABAergic synapses (Sassoè-Pognetto et al., 1995; Essrich et al., 1998; Sassoè-Pognetto and Fritschy, 2000). Moreover, reducing the expression of gephyrin in cultured neurons or mice results in the selective loss of synaptic localization of $GABA_A$Rs composed of α2βγ2 or α3βγ2 subunits (Essrich et al., 1998; Kneussel et al., 1999). These data indicate that the exact role of gephyrin at synapses is receptor subtype specific. Conversely, however, $GABA_A$Rs are essential for postsynaptic clustering of gephyrin at all synapses regardless of the $GABA_A$R subtype normally present (Essrich et al., 1998; Schweizer et al., 2003; Kralic et al., 2006; Studer et al., 2006; Patrizi et al., 2008).

Receptor-subtype-specific functions of gephyrin may be explained at least in part by different modes of interaction of gephyrin with $GABA_A$Rs. Tretter et al. (2008) described a detergent-sensitive interaction of gephyrin with a hydrophobic motif in the cytoplasmic loop region of the receptor α2 subunit (Figure 1C). Yeast two-hybrid assays further suggest a similar interaction between gephyrin and the α3 subunit (Saiepour et al., 2010). Curiously, however, the gephyrin binding motif of the α2 subunit but not the homologous sequence of the α1 subunit is sufficient to target a heterologous membrane protein to synapses (Tretter et al., 2008). A lower-affinity interaction between $GABA_A$Rs and gephyrin than between glycine receptors and gephyrin is consistent with weaker synaptic confinement of $GABA_A$ than glycine receptors (Lévi et al., 2008).

The Neuroligin-Neurexin Complex

The structural and functional maturation of synapses is critically dependent on synaptic adhesion complexes. One such complex involves a transsynaptic interaction of presynaptic neurexins and postsynaptic neuroligins (Figures 3D, 4, and 5A) (Ushkaryov et al., 1992, 1994; Ichtchenko et al., 1995, 1996; Ullrich et al., 1995; Jamain et al., 2008). Overexpression of different neuroligins in neurons or heterologous cells cocultured with neurons can induce presynaptic development of glutamatergic and GABAergic synapses (Scheiffele et al., 2000; Chih et al., 2005; Chubykin et al., 2007; Dong et al., 2007; Fu and Vicini, 2009). Conversely, β-neurexins presented on beads or overexpressed in heterologous cells can induce the formation of separate postsynaptic GABAergic or glutamatergic hemisynapses in cocultured neurons (Graf et al., 2004). Of special interest is NL2 as it is localized selectively at inhibitory synapses (Graf et al., 2004; Varoqueaux et al., 2004) and required for structural and functional maturation of subsets of GABAergic but not glutamatergic or glycinergic synapses in vivo (Varoqueaux et al., 2006; Gibson et al., 2009; Hoon et al., 2009; Poulopoulos et al., 2009). By contrast, NL3 is found at both glutamatergic and GABAergic synapses (Budreck and Scheiffele, 2007), while NL1 and NL4 are found primarily at glutamatergic (Song et al., 1999) and glycinergic (Hoon et al., 2011) synapses, respectively. A recent report has identified gephyrin as a direct interaction partner of NLs (Poulopoulos et al., 2009). Although this interaction lacks selectivity for NL2, this finding provides important clues, detailed further below, for the mechanisms underlying the selective deposition of gephyrin at inhibitory synapses.

Interactions between postsynaptic NL2 and presynaptic neurexins are thought to contribute to proper alignment of pre- and postsynaptic molecules at inhibitory synapses. Nevertheless, NL2 is dispensable for clustering and synaptic localization of gephyrin in most brain areas (Varoqueaux et al., 2006; Hoon et al., 2009) (except dentate gyrus Jedlicka et al., 2011), suggesting that other so-far-unknown synaptogenic complexes might exist. A trans-synaptic interaction between the postsynaptic dystrophin-associated glycoprotein (DG) complex and presynaptic neurexins might contribute to the structural integrity of a subset of inhibitory synapses (Sugita et al., 2001). The DG complex consists of the peripheral membrane protein α-dystroglycan, the integral membrane spanning protein β-dystroglycan, and the subsynaptic cytoskeletal component dystrophin. However, this complex appears late during synaptogenesis and is present at a subset of GABAergic synapses only (Knuesel et al., 1999). Moreover, the DG complex is dispensable for postsynaptic clustering of $GABA_A$Rs and unable to promote the accumulation of $GABA_A$Rs and gephyrin at synapses (Brünig et al., 2002b; Lévi et al., 2002). Recently the synaptic scaffolding and PDZ domain-containing protein S-SCAM (also known as membrane-associated guanylate kinase inverted-2, MAGI-2) was isolated as a β-dystroglycan interacting protein that might physically link the DG complex to NL2 (Sumita et al., 2007). However, S-SCAM also interacts with NL1 and is found at both excitatory and a subset of inhibitory synapses, suggesting an unspecific role in maturation of synapses.

Collybistin

The gephyrin interacting protein collybistin (CB) is a member of the Dbl family of guanine nucleotide exchange factors (RhoGEFs) that selectively activates the small GTPase Cdc42 (Figures 3C, 4, and 5A) (Reid et al., 1999; Kins et al., 2000; Grosskreutz et al., 2001). However, analyses of Cdc42 knockout mice indicate that Cdc42 is dispensable for gephyrin and $GABA_A$R clustering (Reddy-Alla et al., 2010). In neurons, CB is colocalized with gephyrin at inhibitory synapses (Saiepour et al., 2010). When coexpressed with gephyrin in heterologous cells, CB has the unique ability to transform cytoplasmic aggregates of gephyrin into submembrane microclusters that resemble postsynaptic gephyrin clusters of neurons (Kins et al.,

2000). Moreover, CB is required for postsynaptic clustering of gephyrin and $GABA_A$Rs, as shown by analyses of naturally occurring mutations of the CB gene (ARHGEF9) associated with hyperekplexia, epilepsy, and mental retardation in patients (Harvey et al., 2004; Kalscheuer et al., 2009) as well as by CB gene knockout in mice (Papadopoulos et al., 2007). Loss of gephyrin and $GABA_A$R clusters in CB knockout mice is most pronounced in the hippocampus and amygdala. However, in brainstem, neocortex, and several other brain areas the clustering of gephyrin and $GABA_A$ or glycine receptors is unaffected, indicating that other GEFs can compensate for the loss of CB function in these brain areas.

Multiple CB splice variants exist that differ in their C-terminal structures and by the presence or absence of an N-terminal SH3 domain (Kins et al., 2000; Harvey et al., 2004). Intriguingly, the predominant CB isoforms detected in vivo contain an SH3 domain, which inhibits the aforementioned CB-dependent formation of submembrane gephyrin clusters, indicating that CB is negatively regulated by its SH3 domain (Kins et al., 2000; Harvey et al., 2004). However, cotransfection of CB^{SH3+} and gephyrin with NL2 negates the inhibitory effect of the SH3 domain (Poulopoulos et al., 2009). CB splice variants invariably contain a pleckstrin homology (PH) domain that is required for its interaction with plasma-membrane-restricted phosphoinositides and for clustering of gephyrin at inhibitory synapses (Harvey et al., 2004; Reddy-Alla et al., 2010). The data are consistent with a heterotrimeric membrane-associated complex that consists of NL2, CB^{SH3+}, and gephyrin and that enables the selective deposition of gephyrin at NL2-containing inhibitory synapses. Experiments in heterologous cells indicate that NL1 can potentially substitute for NL2 and similarly induce submembrane gephyrin clusters but only with constitutively active CB isoforms that lack an SH3 domain. In addition, preliminary evidence suggests that the α2 subunit can substitute for NL2 and activate the gephyrin-clustering function of CB^{SH3+} (Saiepour et al., 2010). This $GABA_A$R-dependent function of CB is specific for α2-containing receptors and abolished by a naturally occurring missense mutation (CB^{G55A}) that disrupts the clustering of α2-containing $GABA_A$Rs and gephyrin in cultured neurons and is associated with mental retardation, epilepsy, and hyperekplexia in a patient (Harvey et al., 2004; Saiepour et al., 2010). NL1- and α2 subunit-mediated activation of CB might contribute to residual clustering of gephyrin seen in NL2 KO mice (Jedlicka et al., 2011).

Other gephyrin binding proteins that are concentrated at synapses include the Ser/Thr kinase mTor (mammalian target of rapamycin, also known as RAFT1 and FRAP1) (Sabatini et al., 1999) and the dynein light chains (DLC) 1 and 2 (Fuhrmann et al., 2002). mTor functions as an important regulator of mRNA translation, allowing for speculation that gephyrin might contribute to translational control of postsynaptic protein synthesis. This idea is supported by recent evidence that both gephyrin and collybistin are part of the eukaryotic translation initiation factor 3 complex (Sertie et al., 2010). However, whether gephyrin and collybistin play a role in translational control in dendrites remains to be elucidated. The interaction between gephyrin and the DLC is implicated in retrograde vesicular transport of gephyrin-glycine receptor complexes from glycinergic synapses (Maas et al., 2009). The significance of this interaction for $GABA_A$Rs is unclear as intracellular $GABA_A$R-gephyrin-DLC complexes have not been described and the DLC-gephyrin interaction is dispensable for normal localization of gephyrin at GABAergic synapses (Fuhrmann et al., 2002).

Radixin-Mediated Extrasynaptic Clustering of α5βγ2 Receptors

Among different γ2-containing $GABA_A$Rs the α5βγ2 receptors are unique in that they are localized mostly extrasynaptically, as mentioned earlier. Interestingly, even extrasynaptic α5βγ2 receptors are clustered at the plasma membrane (Christie and de Blas, 2002) (Figure 5B). Loebrich et al. (2006) have identified radixin as a α5 subunit-interacting protein that is essential for extrasynaptic clustering of α5βγ2 receptors. Radixin is a member of the ERM (ezrin, radixin, moesin) family of proteins, which are known to link transmembrane proteins to the actin cytoskeleton. Transfection of neurons with a dominant-negative radixin construct abolishes the clustering of α5-containing receptors but does not affect $GABA_A$R surface expression nor GABAergic tonic and phasic currents (Loebrich et al., 2006). The data suggest that radixin-independent mechanisms prevent α5-containing receptors from accumulation at synapses. The functional relevance of α5βγ2 receptor clustering in the extrasynaptic membrane is not known.

Regulation of GABAergic Transmission by Changes in Lateral Diffusion Dynamics of $GABA_A$Rs

Postsynaptic $GABA_A$R clusters represent diffusional confinement areas containing laterally mobile $GABA_A$Rs stabilized by gephyrin. Fluorescence recovery after photobleaching (FRAP) was used to compare the mobility of fluorescently tagged $GABA_A$Rs at postsynaptic and extrasynaptic plasma membrane sites (Jacob et al., 2005). These experiments revealed significantly greater fluorescence recovery rates at extrasynaptic than postsynaptic membrane domains, thereby indicating greater mobility of extrasynaptic than postsynaptic $GABA_A$Rs (Figure 5B). Moreover, the fluorescence recovery rate at the periphery of the photobleached area was greater than that at the center, consistent with replenishment of $GABA_A$Rs from within the plane of the plasma membrane, rather than by insertion into the plasma membrane from intracellular receptor pools. To assess the role of gephyrin in modulating lateral diffusion, FRAP experiments were combined with RNAi knockdown of gephyrin, a treatment that effectively reduced the expression of gephyrin but did not affect the accumulation of $GABA_A$Rs at the plasma membrane. Interestingly, postsynaptic $GABA_A$Rs of gephyrin-RNAi-treated neurons showed significantly greater FRAP recovery rates than control neurons, indicating that the mobility of $GABA_A$Rs at postsynaptic sites is restrained by direct or indirect interactions with gephyrin (Jacob et al., 2005). An independent study relied on an ingenious method to mutate and functionally tag $GABA_A$Rs such that they are permanently inactivated by an inhibitor after receptor activation by GABA (Thomas et al., 2005). This study showed that postsynaptic $GABA_A$Rs are subject to rapid constitutive exchange with nonsynaptic receptor pools without measurable contribution by exocytosis of $GABA_A$Rs from intracellular pools.

NMDAR- and Calcineurin-Mediated Long-Term Depression of GABAergic Synapses

Experiments that tracked the lateral movement of quantum dot-labeled, single $GABA_AR$ molecules showed that the diffusion coefficient of postsynaptic receptors is about half of that of nonsynaptic receptors (Bannai et al., 2009). Increasing neural activity with a K^+-channel blocker increased the diffusion coefficient of both synaptic and extrasynaptic $GABA_ARs$ and decreased the postsynaptic cluster size of gephyrin and $GABA_ARs$, concomitant with a reduction in the amplitude of mIPSCs (Figure 5B). This effect of increased neural activity was dependent on Ca^{2+} influx and activation of calcineurin, did not involve receptor internalization, and was reversed when normal neural activity was restored. These results are consistent with EPSC-induced long-term depression (LTD) of unitary IPSCs observed in association with high-frequency stimulation-induced LTP of the Schaffer collateral-CA1 pathway (Lu et al., 2000; Wang et al., 2003a). LTD of IPSCs required NMDA receptor-dependent recruitment of calcineurin to the $GABA_AR$ complex and calcineurin-mediated dephosphorylation of S327 of the $\gamma2$ subunit. The findings by Wang et al. (2003a) and Bannai et al. (2009) were confirmed by a recent study that combined live imaging of fluorescently tagged $GABA_AR$ clusters with single-molecule tracking of quantum dot-labeled single $GABA_AR$ molecules (Muir et al., 2010). As expected, glutamate-induced dispersal of $GABA_AR$ clusters and enhancement of $GABA_AR$ mobility was critically dependent on NMDA receptor and calcineurin activation and independent of dynamin and therefore did not involve endocytosis of $GABA_ARs$. Moreover, Glu-induced and calcineurin-mediated dephosphorylation of $\gamma2$ S327 increased the lateral mobility and reduced the synaptic residency time of quantum dot-labeled single $GABA_AR$ molecules (Muir et al., 2010) (Figure 5B). Future experiments will need to address how $\gamma2$ S327 regulates interaction of $GABA_ARs$ with the synaptic protein scaffold.

The NMDAR- and calcineurin-mediated form of LTD of inhibitory synapses (Wang et al., 2003a; Bannai et al., 2009; Muir et al., 2010) at first seems in conflict with the aforementioned NMDAR-mediated potentiation of mIPSCs (Marsden et al., 2007). However, more recent evidence suggests that opposite functional effects observed in these two sets of experiments reflect different neuronal stimulation intensities (Marsden et al., 2010). NMDAR-dependent LTD of hippocampal pyramidal cells associated with calcineurin-dependent diffusional dispersal of $GABA_ARs$ reflects robust stimulation of both NMDA and AMPA receptors achieved either by high-frequency stimulation of glutamatergic afferents or by treatment of neurons with K^+-channel blockers or glutamate (Figure 5B). These conditions result in activation of both CaMKII and calcineurin. However, calcineurin inhibits the targeting of CaMKII to inhibitory synapses (Marsden et al., 2010). By contrast, NMDAR-mediated membrane insertion of $GABA_ARs$ and potentiation of mIPSCs is observed following more moderate chemical stimulation of NMDARs only (Figure 5C). Moderate stimulation of neurons with NMDA seemingly fails to activate calcineurin and thereby allows the activation and translocation of CaMKII to inhibitory synapses (Marsden et al., 2010). As mentioned earlier, NMDAR-induced de novo insertion of $GABA_ARs$ into the plasma membrane is further dependent on GABARAP, NSF, and GRIP (Marsden et al., 2007). Thus, the directionality of neural activity-induced trafficking of $GABA_ARs$ is strictly stimulus intensity dependent.

Regulation of $GABA_AR$ Trafficking as a Downstream Effector of Extracellular Neuropeptide Cues

Insulin-Regulated Cell Surface Expression of $GABA_ARs$

Signaling by pancreatic insulin is pivotal for the regulation of peripheral glucose and lipid metabolism. However, insulin is also produced in brain (Havrankova et al., 1981; Stevenson, 1983) and released from neurons in an activity-dependent manner (Clarke et al., 1986). Signaling by insulin receptors contributes to structural maturation of neuronal dendrites, as well as functional synaptic plasticity (reviewed in Chiu and Cline, 2010). In addition, insulin signaling leads to a rapid increase in the cell surface accumulation and function of postsynaptic $GABA_ARs$ (Wan et al., 1997; Wang et al., 2003b).

A first line of investigation indicates that insulin-induced translocation of $GABA_ARs$ to the cell surface requires activation of the serine-threonine kinase Akt, a primary target of insulin signaling downstream of phosphoinositide 3 kinase (PI3K, Figure 6A) (Wang et al., 2003b). PI3K-mediated phosphorylation of membrane lipids is established as a mechanism that leads to recruitment of Akt to the plasma membrane where it is phosphorylated and activated by the serine-threonine kinase, phosphoinositide-dependent kinase 1 (PDK1) (Cantley, 2002). In vitro assays showed that activated Akt phosphorylates a conserved phosphorylation site present in all three β subunits of $GABA_ARs$ (S409 in $\beta1$, S410 in $\beta2$, S408/409 in $\beta3$) (Wang et al., 2003b; Xu et al., 2006). Cotransfection of Akt with $\alpha2\beta2\gamma2$ receptors increased the cell surface expression of these receptors in HEK293 cells. Lastly, phosphorylation of $\beta2$ S410 was shown to be essential for Akt-induced surface expression of corresponding receptors in transfected neurons (Wang et al., 2003b). Curiously, the Akt phosphorylation site of $\beta1$-3 subunits is identical with the aforementioned motif in β subunits that regulates clathrin-mediated endocytosis of $GABA_ARs$. One might therefore conclude that insulin-induced surface expression and function of $GABA_ARs$ reflects reduced clathrin-mediated endocytosis of $GABA_ARs$. However, insulin-induced potentiation of GABA-evoked currents was completely abolished by pretreatment of neurons with brefeldin A (BFA), an inhibitor of anterograde transport from ER to Golgi (Fujii et al., 2010). In the presence of BFA, insulin induced a modest run-down of GABA-evoked currents, thereby facilitating rather than inhibiting $GABA_AR$ endocytosis. These results are consistent with Akt-mediated insertion of newly synthesized $GABA_ARs$ (and to a lesser extent increased endocytosis of $GABA_ARs$). Together with the aforementioned studies by Kittler et al. (2005), these findings indicate that phosphorylation of a single site in $GABA_AR$ β subunits can have different effects on trafficking of $GABA_ARs$ depending on the kinase involved, most likely reflecting different subcellular compartments where phosphorylation of $GABA_ARs$ occurs. Interestingly, Fujii et al. (2010) found that the effects of insulin on GABA-evoked currents are absent in neurons from PRIP1/2 double knockout mice. PRIP1 interacts with Akt directly and is required for insulin-induced association of Akt with $GABA_ARs$. Thus, PRIP serves

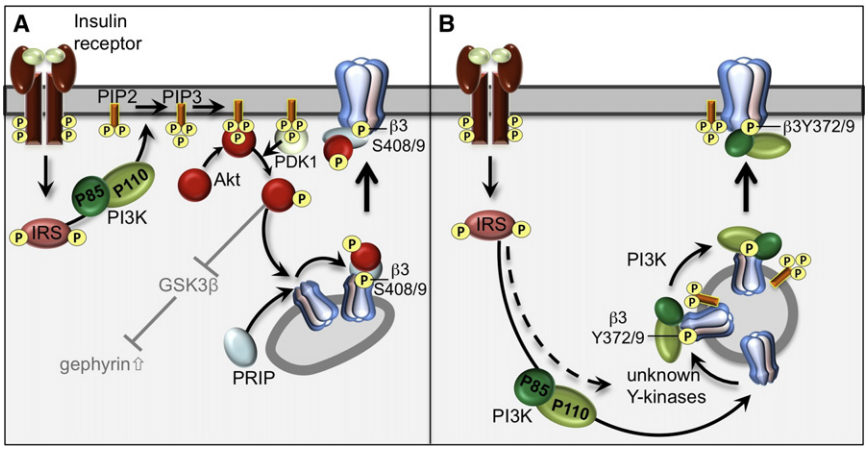

Figure 6. Insulin Receptor-Mediated Surface Expression of $GABA_A$Rs

Two distinct mechanisms have been proposed for insulin-induced surface expression of $GABA_A$Rs that involve either Ser or Tyr phosphorylation of the $GABA_A$R β subunit, respectively, and may function independently or cooperatively.

(A) In a first mechanism, insulin-receptor-mediated phosphorylation of the insulin receptor substrate-1 (IRS-1) leads to activation of PI3K and accumulation of phosphoinositide (PIP3) at the plasma membrane. PIP3-mediated recruitment of Akt to the plasma membrane facilitates PDK1-mediated phosphorylation and activation of Akt. Phosphorylated Akt forms a ternary complex with PRIP and vesicular $GABA_A$Rs, which causes phosphorylation (β3S408/9) and translocation of $GABA_A$Rs to the plasma membrane. As an additional primary downstream target of insulin, Akt is known to inhibit GSK3β. This kinase in turn phosphorylates gephyrin, which triggers calpain-1-mediated degradation of gephyrin. Thus, insulin signaling may promote GABAergic synaptic function by increasing the surface expression and gephyrin-dependent synaptic confinement of $GABA_A$Rs.

(B) In an alternate mechanism, the PI3K P85 subunit interacts directly with $GABA_A$Rs. This complex is abundant already under basal condition and dependent on phosphorylated Tyr residues (Y372/9) of the $GABA_A$R β3 subunit. On stimulation with insulin, the abundance of this complex and its association with phosphorylated lipids (PIP3) is rapidly increased, concurrent with translocation of the complex to the plasma membrane.

as a multifunctional adaptor for both kinases and phosphatases and thereby contributes to both regulated membrane translocation and endocytosis of $GABA_A$Rs (Fujii et al., 2010). Interestingly, insulin-mediated activation of Akt further results in inhibitory serine-phosphorylation of GSK3β (Cross et al., 1995). GSK3β in turn promotes postsynaptic $GABA_A$R clustering and mIPSCs by reducing calpain-1-mediated cleavage of gephyrin (Tyagarajan et al., 2011) (Figures 5A and 6A).

A second proposed mechanism involves direct interaction of the insulin receptor target PI3K with $GABA_A$Rs (Figure 6B) (Vetiska et al., 2007). The PI3K-$GABA_A$R complex was found to be present constitutively in brain tissue, presumably in an intracellular inactive state. When brain slices were treated with insulin the abundance of this complex rapidly increased as well as its association with phosphorylated membrane lipids. This suggests that the complex is translocated to the plasma membrane in concert with PI3K-mediated phosphorylation of lipids. In vitro analyses revealed that the PI3K-$GABA_A$R complex involves binding of the PI3K p85 subunit SH2 domain to phospho-tyrosines (Tyr 372/379) in the intracellular loop region of β subunits. These Tyr residues were essential for insulin-induced surface expression of β2-containing receptors in transfected neurons. However, several aspects of this mechanism remain to be resolved. First, it is unclear whether this mechanism applies to $GABA_A$Rs independently of the type of β subunit. Second, it is not known whether Tyr phosphorylation of β subunits is itself modulated by insulin, which Tyr kinase is involved in β subunit phosphorylation, and whether interaction of PI3K with β subunit phospho-tyrosines contributes to activation of PI3K enzyme function. Lastly, it is not known whether and how insulin-induced interaction between PI3K and $GABA_A$Rs corroborates with the aforementioned Akt- and PRIP-dependent downstream effects of PI3K.

BDNF-Regulated Trafficking of $GABA_A$Rs

Signaling by BDNF and its cognate receptor (receptor tropomyosin-related kinase B, TrkB) is critically important for neurogenesis (Bergami et al., 2008; Li et al., 2008) and inhibitory synapse formation (Seil, 2003). BDNF is further involved in structural and functional neuronal plasticity in the adult nervous system (Xu et al., 2000). At GABAergic synapses, BDNF leads to a rapid and transient increase followed by a lasting reduction in the amplitude of mIPSCs (Brünig et al., 2001; Jovanovic et al., 2004). The lasting reduction in mIPSC amplitude is correlated with reduced surface expression of $GABA_A$Rs (Brünig et al., 2001). Mechanistically, BDNF-induced up- and downregulation of mIPSCs involves a biphasic modulation of the Ser408/409 phosphorylation state of β3 subunits (Jovanovic et al., 2004). Initial rapid phosphorylation is correlated with a transient association of $GABA_A$Rs with PKC and the receptor for activated C-kinase (RACK-1). Subsequent dephosphorylation of the β3 subunit is predominantly mediated by PP2A. As discussed earlier, dephosphorylation of β3 Ser408/409 by PP2A promotes the association of $GABA_A$Rs with AP2, which in turn facilitates clathrin-mediated endocytosis of $GABA_A$Rs (Kittler et al., 2005) and explains the lasting effects of BDNF on $GABA_A$Rs surface expression and mIPSCs. Interestingly, the recruitment of PP2A to $GABA_A$Rs is critically dependent on the phosphatase adaptor PRIP (Kanematsu et al., 2006). Treatment of hippocampal PRIP1/2 double knockout neurons with BDNF resulted in a steady rise in β3 phosphorylation accompanied by increased GABAergic whole-cell currents, indicating that PKC-mediated phosphorylation remained intact while the subsequent PRIP-dependent and PP2A-mediated dephosphorylation step was disrupted (Kanematsu et al., 2006). Thus, PRIP plays essential roles both in BDNF-induced downregulation and insulin-induced potentiation of GABAergic postsynaptic function.

Wnt-Regulated Cell Surface Expression of $GABA_A$Rs

Wnt signaling is critically involved in diverse aspects of embryonic development, neural differentiation, and adult synaptic plasticity (reviewed by Inestrosa and Arenas, 2010; Budnik and Salinas, 2011). Wnt proteins encoded by 19 different genes act through several different frizzled family receptors to induce

multiple signal transduction pathways. The canonical Wnt pathway involves inhibition of GSK3β in the axin/GSK3β/APC complex, which leads to accumulation and nuclear translocation of β-catenin and activation of β-catenin-dependent gene expression. By contrast, two noncanonical Wnt pathways activate either c-Jun N-terminal kinase (Wnt/JNK pathway) or CaMKII (Wnt/Ca^{2+} pathway) as downstream targets. All three pathways are implicated in the regulation of synaptic plasticity, primarily of excitatory synapses and both pre- and postsynaptically (Inestrosa and Arenas, 2010). In addition, Wnt-5a was recently shown to result in rapid (5 min) and significant (+40%) upregulation of $GABA_AR$ clusters in cultured neurons (Cuitino et al., 2010). This effect was due to postsynaptic changes as it was paralleled by increased amplitudes but not frequency of mIPSCs recorded from cultured neurons. Consistent with this interpretation, the time course and paired-pulse relationship of evoked IPSCs recorded from hippocampal slices were unaffected by Wnt-5a. The effect of Wnt-5a on mIPSCs was further potentiated by blockade of endocytosis with a dynamin inhibitor peptide. In addition, Wnt-5a treatment reduced the pool of previously surface biotinylated and internalized $GABA_ARs$, suggesting that increased clustering of $GABA_ARs$ reflected enhanced recycling of endocytosed receptors. In support of this mechanism, treatment of neurons with a Wnt-5a-mimicking peptide (Foxy-5) that specifically activates noncanonical Wnt pathways replicated the Wnt-5a effect on $GABA_AR$ clustering. Moreover, cotreatment with Foxy-5 and pathway-specific pharmacological inhibitors allowed the conclusion that Wnt-5A-induced clustering of $GABA_ARs$ involved the noncanonical Wnt/Ca^{2+} pathway and CaMKII. The CaMKII targets that are phosphorylated in response to Wnt-5a have so far not been determined. In addition to the Wnt/Ca^{2+} pathway the canonical Wnt pathway is strongly implicated in the regulation of GABAergic inhibition by the aforementioned effects of Li^+ and GSK3β on the stability and postsynaptic clustering of gephyrin (Tyagarajan et al., 2011). However, in apparent conflict with this study, the canonical Wnt ligand Wnt-7A and Li^+ had no significant effect on $GABA_AR$ clustering in the study by Cuitino et al. (2010).

Conclusions and Outlook

There has been remarkable progress in understanding the mechanisms that regulate GABAergic transmission. Dynamic changes in $GABA_AR$ trafficking represent prevalent forms of GABAergic neural plasticity, although changes in subunit gene expression, Cl^- reversal potential, and GABA release are also important, especially under pathological conditions. $GABA_AR$-associated proteins and signaling factors involved in $GABA_AR$ trafficking are shared with other signal transduction pathways, thereby allowing for complex interactions among multiple neurotransmitter and signaling systems.

Developmental imbalances between neural excitation and inhibition are broadly implicated in the etiology of the most prevalent neuropsychiatric disorders. Such imbalances may be further amplified by trafficking deficits in $GABA_ARs$, as suggested by activity and anoxia-induced loss of postsynaptic $GABA_ARs$ (Mielke and Wang, 2005; Terunuma et al., 2008; Arancibia-Cárcamo et al., 2009). Indeed, deficits in GABAergic transmission may be central to the etiology of neuropsychiatric disorders such as major depressive disorder (Luscher et al., 2011), bipolar disorder (Craddock et al., 2010), and schizophrenia (Charych et al., 2009). Conversely, the cell surface trafficking and synaptic accumulation of $GABA_ARs$ is modulated by Wnt pathway kinases (GSK3β, Akt) that are central to the therapeutic action of mood stabilizing and antidepressant drugs (Logan and Nusse, 2004; Okamoto et al., 2010; Tyagarajan et al., 2011). Further progress in understanding of $GABA_AR$ trafficking mechanisms should provide better mechanistic insights into these disorders and facilitate the development of more effective drug therapies.

Despite the recent progress, diverse aspects of $GABA_AR$ trafficking remain poorly understood. For example, current understanding of trafficking mechanisms largely fails to account for the structural heterogeneity of $GABA_ARs$. We can rationally explain the postsynaptic accumulation of $\alpha2\beta\gamma2$ and $\alpha3\beta\gamma2$ receptors. By contrast the postsynaptic clustering of $\alpha1\beta\gamma2$ receptors can occur in the absence of gephyrin and therefore probably depends on alternative mechanisms. Disruption of Tyr phosphorylation in $\gamma2^{Y365/7F}$ knockin mice results in selective upregulation of $GABA_ARs$ in CA3 pyramidal cells but, so far unexplained, not in CA1 pyramidal cells (Tretter et al., 2009). Phosphorylation of $\gamma2^{S327}$ is established to modulate the diffusional dynamics of postsynaptic $GABA_ARs$ (Muir et al., 2010), yet the functionally relevant interaction partner(s) for this effect remain unknown. Lastly, gephyrin exists in multiple splice variants (Paarmann et al., 2006) and is phosphorylated at multiple sites (Huttlin et al., 2010; Tyagarajan et al., 2011), yet the functional relevance of different gephyrin isoforms and their posttranslational modifications remain largely unexplored.

One attractive mechanism underlying postsynaptic differentiation involves gephyrin-mediated interaction of $GABA_ARs$ with NL2, which accumulates at synapses through interaction with presynaptic neurexin. However, the loss of $GABA_ARs$ and gephyrin from inhibitory synapses of NL2 knockout mice is incomplete (Hoon et al., 2009; Jedlicka et al., 2011), suggesting that additional thus-far-unidentified synaptic adhesion complexes exist that substitute for NL2 and contribute to accumulation of $GABA_ARs$ and gephyrin at many synapses. Interestingly, a recently described transsynaptic interaction between presynaptic neurexins and postsynaptic $GABA_ARs$ appears to inhibit rather than promote the function of GABAergic synapses (Zhang et al., 2010). This negative effect of neurexin on GABAergic transmission was preserved in NL2 KO neurons and also observed in $GABA_AR$ expressing heterologous cells exposed to soluble neurexin constructs, indicating that it does not involve competition between $GABA_ARs$ and NL2 for interaction with neurexin. The relevance of this interaction for native synapses and trafficking of $GABA_ARs$ remains to be explored.

A complete understanding of the role of $GABA_AR$ trafficking in GABAergic synaptic plasticity will require that these knowledge gaps be filled. In addition, downstream consequences of altered GABAergic transmission on other signaling pathways will need to be explored. Ultimately, the function of these mechanisms will need to be explored at the level of neural network activity, behavior, and cognition, including in appropriate disease models.

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