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Molecular diversity, trafficking and subcellular localization of $GABA_B$ receptors

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Abstract

$GABA_B$ receptors are the G-protein coupled receptors for the main inhibitory neurotransmitter in the brain, γ-aminobutyric acid (GABA). While native studies predicted pharmacologically distinct $GABA_B$ receptor subtypes, molecular studies failed to identify the expected receptor varieties. Mouse genetic experiments therefore addressed whether the cloned receptors can account for the classical electrophysiological, biochemical and behavioral $GABA_B$ responses or whether additional receptors exist. Among G-protein coupled receptors, $GABA_B$ receptors are unique in that they require 2 distinct subunits for functioning. This atypical receptor structure triggered a large body of work that investigated the regulation of receptor assembly and trafficking. With the availability of molecular tools, substantial progress was also made in the analysis of the receptor protein distribution in neuronal compartments. Here, we review recent studies that shed light on the molecular diversity, the subcellular distribution and the cell surface dynamics of $GABA_B$ receptors.

Keywords: $GABA_B$; Compartmentalization; Endocytosis; Desensitization; Tolerance

Abbreviations: ARF, ADP-ribosylation factor; ATF4, activating transcription factor 4; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein; COPI, coat protein I complex; CREB2, cAMP response element binding-protein 2; PKA, cyclic AMP (cAMP)-dependent protein kinase; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; GEF, guanine-nucleotide-exchange factor; GHB, γ-hydroxybutyrate; GPCR, G-protein coupled receptors; GRK, G-protein receptor kinases; PSD, postsynaptic density; sIPSC, slow inhibitory postsynaptic current; TGN, *trans*-Golgi network.

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1. Introduction

Metabotropic $GABA_B$ receptors were first identified based on the receptor's distinct pharmacological profile compared to ionotropic $GABA_{A/C}$ receptors (Hill & Bowery, 1981). It was

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subsequently shown that $GABA_B$ receptors are G-protein coupled receptors (GPCR) that inhibit adenylyl cyclase via the $G_{\alpha i/o}$ subunits of the activated G-protein (Hill, 1985). The physiological consequences of inhibiting adenylyl cyclase activity via $GABA_B$ receptors are poorly understood but include effects on transcription factors (Steiger et al., 2004) and kinases (Diverse-Pierluissi et al., 1997; Couve et al., 2002; Ren & Mody, 2003). Biochemical experiments revealed that $GABA_B$ receptors control transmitter release at several synapses. Retrospectively, $GABA_B$ receptors were mostly studied using electrophysiological techniques. These studies showed that $GABA_B$ receptors regulate Ca^{2+}- and K^+-channels at pre- and postsynaptic sites via the $G_{\beta\gamma}$ subunits (Bowery et al., 2002; Calver et al., 2002; Bettler et al., 2004). Presynaptic $GABA_B$ receptors are present on inhibitory and excitatory terminals where they function as auto- and heteroreceptors, respectively. Stimulation of presynaptic $GABA_B$ receptors suppresses neurotransmitter release by inhibition of voltage-sensitive Ca^{2+}-channels (Mintz & Bean, 1993; Thompson et al., 1993; Poncer et al., 1997), but a direct modulation of synaptic vesicle priming was also proposed (Sakaba & Neher, 2003). Postsynaptic $GABA_B$ receptors induce a slow inhibitory postsynaptic current (sIPSC) by gating Kir3-type K^+-channels, which hyperpolarizes the membrane and shunts excitatory currents (Lüscher et al., 1997). $GABA_B$ receptors have also been implicated in the modulation of synaptic plasticity (Davies et al., 1991; Patenaude et al., 2003; Huang et al., 2005), heterosynaptic depression (Vogt & Nicoll, 1999), population burst firing and inhibition of backpropagating action potentials (Zilberter et al., 1999; Leung & Peloquin, 2006). From these studies it emerges that $GABA_B$ receptors are not only present at synaptic sites, but also on extrasynaptic membranes of axons and dendrites.

$GABA_B$ receptors are considered promising drug targets (Bettler et al., 2004; Cryan & Kaupmann, 2005; Bowery, 2006). A large body of preclinical data for a host of indications is available for the $GABA_B$ agonist baclofen. However, the use of baclofen in humans has been limited owing to the rapid development of tolerance and adverse effects following systemic administration. It is hoped that novel structural classes of compounds will overcome the shortcomings of available $GABA_B$ agonists. In that respect, significant progress was made in identifying positive allosteric modulators that are devoid of the side-effects observed with classical $GABA_B$ agonists (Urwyler et al., 2001; Urwyler et al., 2003; Cryan et al., 2004). $GABA_B$ antagonists carry a proconvulsive liability, which prevented rapid drug development (Vergnes et al., 1997). Nonetheless, Phase II trials were initiated with the low-affinity antagonist CGP36742 (renamed to SGS742) in patients suffering from mild cognitive impairment and mild-moderate Alzheimer disease (Froestl et al., 2004). No statistically significant improvements were observed between Alzheimer disease patients and the placebo group. However, a detailed analysis of individual patient groups included in the trial suggests cognitive improvements in the mild Alzheimer disease subpopulation (Tomlinson et al., 2004). A large body of work on native receptors suggests the existence of pharmacologically distinct receptor subtypes, which would allow a more selective therapeutic interference with the $GABA_B$ system (Bonanno & Raiteri, 1993; Gemignani et al., 1994; Cunningham & Enna, 1996; Deisz et al., 1997; Mohler & Fritschy, 1999; Yamada et al., 1999; Bowery et al., 2002). However, the proposed pharmacological heterogeneity is not readily supported by molecular studies (Bettler et al., 2004). From a drug discovery point of view, it is therefore crucial to clarify whether additional proteins that generate functional or pharmacological diversity in the $GABA_B$ system exist. Similarly, it is important to understand the dynamics of $GABA_B$ receptors in response to pharmacological treatment. Here, we review recent studies that addressed these issues.

2. Molecular diversity

Based on considerable evidence from native work, most researchers in the field expected the existence of a multitude of $GABA_B$ receptor subtypes with specific subcellular distributions and effector systems, analogous to the metabotropic glutamate receptors that belong to the same gene family (Conn & Pin, 1997). However, only the existence of 2 molecular subtypes of $GABA_B$ receptors is firmly established (Bettler et al., 2004). It is now well accepted that $GABA_B$ receptors assemble into heteromers composed of a $GABA_{B1}$ and a $GABA_{B2}$ subunit, which are both required for normal receptor functioning (Marshall et al., 1999; Mohler & Fritschy, 1999; Bettler et al., 2004). Recombinant experiments support that interaction of $GABA_{B1}$ with $GABA_{B2}$ subunits is not only mandatory for cell surface expression but also for G-protein coupling (Margeta-Mitrovic et al., 2000; Calver et al., 2001; Galvez et al., 2001; Margeta-Mitrovic et al., 2001; Pagano et al., 2001; Robbins et al., 2001; Duthey et al., 2002; Havlickova et al., 2002). Molecular diversity in the $GABA_B$ system arises from the $GABA_{B1a}$ and $GABA_{B1b}$ subunit isoforms (Kaupmann et al., 1997). Other reported $GABA_{B1}$ cDNA isoforms are either not conserved among different species or the existence of stable protein products in vivo could not be demonstrated. Two cDNA isoforms were described for $GABA_{B2}$. The transcripts for these isoforms do not match consensus sequences for splice junctions, supporting that they represent cloning artifacts (Martin et al., 2001). A large body of work indicates that the $GABA_{B1a}$ and $GABA_{B1b}$ isoforms exhibit no unique pharmacological or functional properties in heterologous expression systems. Isolated reports proposed the existence of subunit differences (Leaney & Tinker, 2000; Ng et al., 2001; Parker et al., 2004), but these could not be reproduced by others (Lanneau et al., 2001; Patel et al., 2001; Jensen et al., 2002; Shimizu et al., 2004). Most, if not all, neurons in the brain co-express $GABA_{B1a}$ and $GABA_{B1b}$, which are generated by differential promoter usage from the *$GABA_{B1}$* gene (Bischoff et al., 1999; Steiger et al., 2004). Structurally, the isoforms differ in their N-terminal ectodomain by a pair of sushi repeats that is present in $GABA_{B1a}$ but not in $GABA_{B1b}$ (Blein et al., 2004). Sushi repeats, also known as complement control protein modules, or short consensus repeats are found in other GPCR as well (Grace et al., 2004) and mediate protein interactions in a wide variety of

adhesion proteins (Lehtinen et al., 2004). Since the proposed heterogeneity of native $GABA_B$ receptors is difficult to reconcile with the discovery of only 2 structurally distinct receptors, it was suggested that the sushi repeats in $GABA_{B1a}$ bind to auxiliary proteins that modify receptor activity or pharmacology in vivo (Marshall et al., 1999; Mohler & Fritschy, 1999). Interestingly, the 2 sushi repeats in $GABA_{B1a}$ exhibit strikingly different structural properties (Blein et al., 2004). This led to the proposal that they participate in protein interactions with multiple partners, which could generate some of the heterogeneity predicted by studies with native $GABA_B$ receptors.

In the light of conflicting data from recombinant and native studies regarding $GABA_B$ receptor heterogeneity, mouse genetic studies have addressed whether the cloned subunits can account for classical $GABA_B$ functions in vivo. Mice with ablations in the *GABA$_{B1}$* (lacking both $GABA_{B1a}$ and $GABA_{B1b}$ subunits) and *GABA$_{B2}$* genes reveal a complete absence of typical biochemical, electrophysiological and behavioral $GABA_B$ responses, which renders the existence of additional obligatory receptor subunits unlikely (Prosser et al., 2001; Schuler et al., 2001; Quéva et al., 2003; Gassmann et al., 2004). The predominantly heteromeric nature of native $GABA_B$ receptors is emphasized by the substantial down-regulation of $GABA_{B1}$ and $GABA_{B2}$ protein in the $GABA_{B2}$- and $GABA_{B1}$-deficient mice, respectively. This supports that in native tissues, the interaction between the $GABA_{B1}$ and $GABA_{B2}$ proteins prevents their degradation. $GABA_B$ knock-out mice also revealed whether $GABA_{B1}$ or $GABA_{B2}$ subunits can generate functional receptors in the absence of their partner subunit in vivo. Small G-protein dependent $GABA_B$ responses were observed in the $GABA_{B2}$-deficient, but not in the $GABA_{B1}$-deficient mice (Gassmann et al., 2004). These residual $GABA_B$ responses are mediated by $GABA_{B1}$ and caused by the inhibition of a constitutively active K^+-channel, which is opposite to the activation of K^+-channels normally observed. It remains unclear whether these atypical $GABA_{B1}$ responses are of physiological relevance or represent an artifact of the knockout situation. Since the temporal and spatial expression of $GABA_{B1}$ and $GABA_{B2}$ subunits do not always match (Bettler et al., 2004), it is possible that functional $GABA_B$ receptors exist in neurons that normally lack $GABA_{B2}$ subunits. Co-immunoprecipitation and fluorescence resonance energy transfer experiments suggest that $GABA_{B1}$ subunits can associate as stable homodimers (Villemure et al., 2005). The $GABA_B$ responses observed in $GABA_{B2}$ knock-out mice may therefore relate to $GABA_{B1}$ homodimers, albeit such homodimers were mostly found to be confined to the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartments.

$GABA_{B1}$ knock-out mice also revealed that the physiological effects of exogenously applied γ-hydroxybutyrate (GHB), a widely used recreational drug (Crunelli et al., 2006), are mediated by $GABA_B$ receptors (Kaupmann et al., 2003). While high-affinity [^{3}H]GHB binding sites persist in the brain of $GABA_B$-deficient mice, these binding sites do not appear to participate in any of the well-known $GABA_B$ receptor mediated responses (Kaupmann et al., 2003; Wu et al., 2004). It is therefore unlikely that high-affinity [^{3}H]GHB binding sites represent a pharmacologically distinct subtype of $GABA_B$ receptors, as was proposed. In summary, genetic studies confirm the data from recombinant studies and support that native $GABA_B$ responses are predominantly mediated by heteromeric receptors derived from the *GABA$_{B1}$* and *GABA$_{B2}$* genes. However, $GABA_{B1}$ subunits have the potential to be functional in the absence of a $GABA_{B2}$ subunit in vivo (Gassmann et al., 2004). This is in contrast to recombinant studies positing that $GABA_{B1}$ subunits do not traffic to the cell surface and do not couple to G-proteins in the absence of a $GABA_{B2}$ subunit.

3. Surface trafficking

$GABA_B$ receptors were the first example of a GPCR that necessarily needs to heterodimerize to function in heterologous cells. Many investigators used this unique property to study the mechanism underlying subunit assembly and receptor surface trafficking (Fig. 1). It is now well established that a C-terminal arginine-based ER retention/retrieval signal, RSRR, retains unassembled $GABA_{B1}$ subunits in the ER and restricts surface expression of correctly assembled heteromeric receptors (Couve et al., 1998; Margeta-Mitrovic et al., 2000; Pagano et al., 2001). Presumably, the RSRR signal in $GABA_{B1}$ is inactivated by interaction of the $GABA_{B1}$ with the $GABA_{B2}$ subunit, which triggers forward trafficking. This is corroborated by transgenic work. Mice expressing a C-terminally truncated $GABA_{B2}$ protein lack functional receptors at the cell surface because the RSRR signal of $GABA_{B1}$ remains exposed in the heterodimer (Thuault et al., 2004). More recently, $GABA_B$ receptor trafficking was studied through the characterization of associated proteins that regulate receptor sorting to intracellular compartments. $GABA_B$ interacting proteins were typically identified in yeast two-hybrid screens using the large C-terminal domains of $GABA_B$ subunits as baits. Most of the proteins identified associate with $GABA_B$ subunits through coiled-coil domain interactions. Recent work indicates an interaction of coat protein I complex (COPI) with the RSRR sequence in $GABA_{B1}$ (Brock et al., 2005). Also known as coatomer, COPI is a complex of 7 subunits (α, β, β′, δ, γ, ε and ζ) divided into 2 subcomplexes, and recruited en bloc to Golgi membranes during retrograde Golgi-ER trafficking (McMahon & Mills, 2004). It is proposed that COPI binding to $GABA_{B1}$ acts as a quality control mechanism that retrieves unassembled $GABA_{B1}$ subunits from the *cis*-Golgi to the ER. C-terminal deletions that position the RSRR signal closer to the membrane drastically reduce its effectiveness (Gassmann et al., 2005), supporting that proximity to the membrane restricts access of COPI to the RSRR motif. 14-3-3 proteins compete for the binding site of COPI at $GABA_{B1}$ subunits (Couve et al., 2001; Brock et al., 2005). The reason for this mutually exclusive interaction is unclear, since 14-3-3 association neither affects trafficking nor signaling of $GABA_B$ receptors. Cell surface expression is not only controlled by the occlusion of the RSRR motif, but through additional motifs that act at distinct checkpoints along the secretory pathway. A guanine-nucleotide-exchange factor (GEF) for the ADP-ribosylation factor

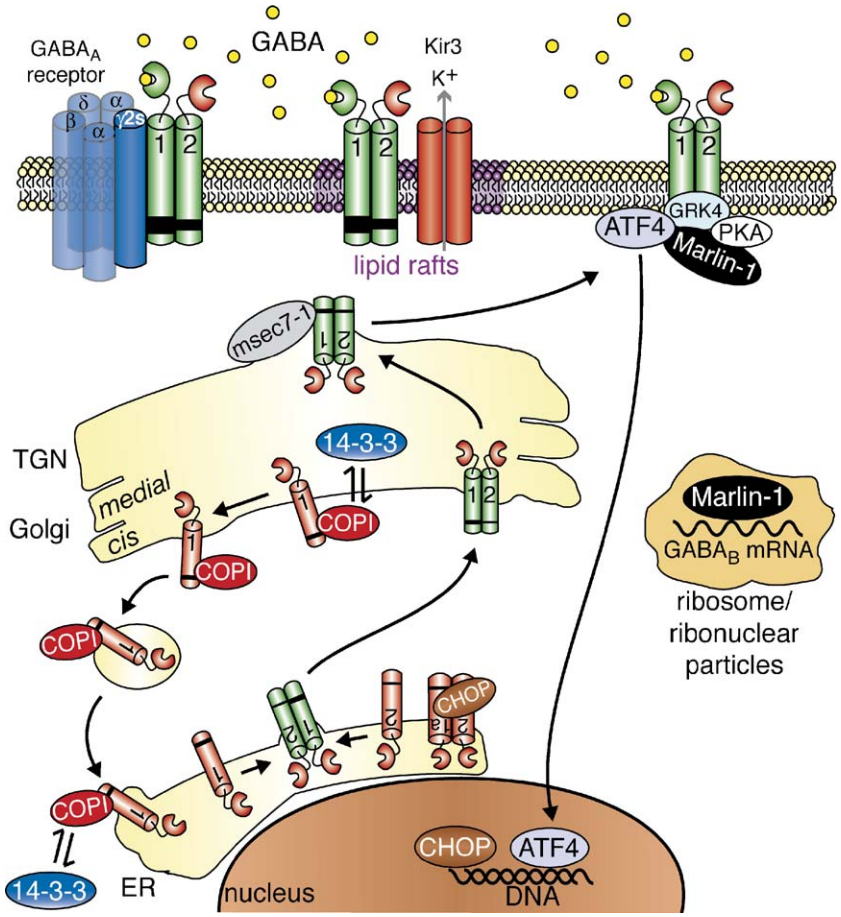

Fig. 1. Regulation of $GABA_B$ receptor trafficking and cell surface expression. Monomeric $GABA_{B1}$ subunits are retained in the ER through the exposure of an RSRR-type retention/retrieval signal in their C-terminal coiled-coil domain (black bar). The RSRR motif is occluded upon interaction with the $GABA_{B2}$ subunit, which is part of a quality control system that ensures that only correctly assembled heterodimeric complexes proceed through the Golgi compartments en route to the plasma membrane. Monomeric $GABA_{B1}$ subunits that escape from the ER to the *cis*-Golgi compartment bind to COPI via the RSRR motif and shuttle back to the ER via COPI-coated vesicles. Members of the 14-3-3 protein family compete with COPI for RSRR binding in the ER and/or the *cis*-Golgi compartment, but do not affect trafficking or function of $GABA_B$ receptors. An LL motif in the coiled-coil domain of $GABA_{B1}$ is important for association with msec7-1, a GEF for multiple ARF proteins. It is postulated that msec7-1 is involved in the efficient unidirectional transport of $GABA_B$ receptors from the TGN to the cell surface, but the precise mechanism is unknown. $GABA_B$ receptors at the cell surface are unusually stable and show little basal or agonist-stimulated endocytosis. A fraction of $GABA_B$ protein and effector Kir3 channels is present in lipid rafts. Segregation of $GABA_B$ receptors into lipid rafts is proposed to alter the composition of associated signaling molecules or to decrease receptor activity. PKA phosphorylation of the $GABA_{B2}$ subunit prevents receptor desensitization and degradation. In cerebellar granule cells, GRK4 is proposed to promote receptor desensitization via a mechanism that does not depend on the kinase domain of GRK4. $GABA_B$ receptors are reported to interact with the transcription factors ATF4 and CHOP. The functional significance of these interactions is unclear, but may include effects on transcription (ATF4) and selective retention of heteromers assembled with the $GABA_{B1a}$ subunit in close proximity to the nucleus (CHOP). The RNA binding protein Marlin-1 interacts with the $GABA_{B1}$ protein but paradoxically controls $GABA_{B2}$ protein levels. In addition, Marlin-1 associates with different species of RNA including those encoding the $GABA_{B1}$ and $GABA_{B2}$ proteins, suggesting that RNA metabolism may form part of the regulatory mechanism involved in $GABA_B$ receptor protein synthesis and trafficking. The γ2S subunit of $GABA_A$ receptors binds to $GABA_{B1}$ subunits and promotes their cell surface expression in the absence of $GABA_{B2}$ subunits. Furthermore, interaction with the γ2S subunit enhances agonist-induced internalization of heteromeric $GABA_B$ receptors.

(ARF) family of GTPases, msec7-1, is proposed to increase cell surface expression through interaction with the LL motif within the cytoplasmic domain of $GABA_{B1}$ (Restituito et al., 2005). The msec7-1 protein acts at the level of the *trans*-Golgi network (TGN), likely by controlling export of $GABA_B$ receptors from this organelle. Although msec7-1 was shown to be involved in the endocytosis of some cell surface proteins, it does not appear to play a role in $GABA_B$ receptor endocytosis. Some of the data obtained with interacting proteins remain controversial. Two transcription factors, activating transcription factor 4 (ATF4)/cAMP response element binding-protein 2 (CREB2) (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001) and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) (Sauter et al., 2005), were reported to associate with $GABA_B$ subunits through coiled-coil interactions. Translocation of ATF4/CREB2 either into or out of the nucleus was seen following $GABA_B$ receptor activation (Vernon et al., 2001; White et al., 2000). The reason for these opposing effects remains unclear, and a physiological significance for the ATF4/CREB2 interaction with $GABA_B$ receptors awaits elucidation. In HEK293 cells, CHOP was reported to selectively accumulate $GABA_{B(1a,2)}$ but not $GABA_{B(1b,2)}$ receptors at intracellular sites, mainly in the ER. This accumulation depends on the interaction of CHOP with 2 distinct domains in the heteromeric

receptor. The C-terminal domains of CHOP and $GABA_{B2}$ associate through coiled-coil interaction, while the N-terminal domain of CHOP associates with an unidentified intracellular site of $GABA_{B1a}$. Since the intracellular domains of $GABA_{B1a}$ and $GABA_{B1b}$ are identical, this subtype-selective interaction is not easily explained and probably involves a conformation-sensitive epitope that is unique to $GABA_{B1a}$. While questions remain, this finding is both intriguing and provocative as it suggests for the first time that the $GABA_B$ receptor subunit composition is a crucial regulator of surface trafficking. The RNA binding protein Marlin-1 is yet another protein that associates with $GABA_{B1}$ through coil coil domain interactions (Couve et al., 2004). Paradoxically, down-regulation of Marlin-1 protein using small interference RNA enhances intracellular $GABA_{B2}$ protein levels without affecting $GABA_{B1}$ protein levels. Marlin-1 also binds different RNA species, including the 3'-untranslated regions of both the $GABA_{B1}$ and $GABA_{B2}$ mRNAs. While it is difficult to conglomerate the interactions of Marlin-1 with both $GABA_B$ subunit mRNA and protein into a coherent mechanistic scheme, Marlin-1 is proposed to have significant effects on the production of functional $GABA_B$ receptor heteromers. Interestingly, Marlin-1 co-fractionates with ribosome and ribonuclear particles, but not with ER markers. It is therefore conceivable that Marlin-1 is present in somatodendritic Golgi outposts (Horton et al., 2005; Horton & Ehlers, 2004), where it could participate in local $GABA_B$ protein synthesis. A recent paper demonstrates that Marlin-1 associates with non-receptor tyrosine kinases that modify microtubule polymers (Steindler et al., 2004). The roles of Marlin-1 in the context of $GABA_B$ receptors may therefore include vesicular trafficking and clearly need further investigation. A physical interaction between $GABA_A$ and $GABA_B$ receptor subunits was also reported (Balasubramanian et al., 2004). Association of $GABA_{B1}$ with the γ2S subunit of $GABA_A$ receptors robustly promotes cell surface expression of $GABA_{B1}$ in the absence of $GABA_{B2}$. The $GABA_{B1}$/γ2S complex is not functional when expressed alone, as assessed by kinase activation experiments and electrophysiological assays. However, the γ2S subunit also associates with the functional $GABA_{B(1,2)}$ heterodimer and markedly enhances $GABA_B$ receptor internalization in response to agonist stimulation. The crosstalk between $GABA_A$ and $GABA_B$ subunits therefore regulates $GABA_B$ receptor trafficking, both in and out of the plasma membrane.

4. Desensitization and internalization

Of special interest are the mechanisms that modulate the function and stability of $GABA_B$ receptors at the cell surface. Desensitization describes the phenomenon whereby the response of a given receptor declines over time despite the continuous presence of the agonist. For many GPCR, desensitization is primarily mediated by G-protein receptor kinases (GRK) and arrestins. GRK specifically bind to the agonist-occupied receptor and catalyze its phosphorylation. This in turn increases the affinity of the receptor for arrestin, thus precluding G-protein interactions and subsequent effector activation. Receptor desensitization and internalization are often interrelated processes, since arrestin binding also leads to the removal of the receptor from the plasma membrane via clathrin-mediated endocytosis. The dynamic control of the cell surface activity and stability of $GABA_B$ receptors is not only of immediate importance for drug discovery but also likely to be of physiological relevance. Several studies therefore addressed whether $GABA_B$ receptors follow the classical agonist-promoted desensitization paradigm and whether desensitization is linked with endocytosis.

A problem of many $GABA_B$ desensitization studies is that the responses from downstream effectors are frequently used to assess desensitization. Therefore, the desensitization properties of the receptor are confounded by the desensitization properties of the effector system. Little information on the desensitization properties of native $GABA_B$ responses is available. In dopaminergic neurons of the ventral tegmental area, $GABA_B$-mediated Kir3 responses desensitize within minutes, whereas the responses from local interneurons do not (Cruz et al., 2004). It is unclear whether the receptor, the effectors or both desensitize in response to the sustained stimulation. The differences in desensitization could relate to a cell-type specific expression of effector Kir3 subunits with different inherent desensitization characteristics. For example, the Kir3.3 subunit contributes to $GABA_B$-mediated postsynaptic inhibition in hippocampal neurons, but not in substantia nigra pars compacta neurons, demonstrating that different Kir3-channel subunit-compositions are linked to $GABA_B$ receptors (Koyrakh et al., 2005). Alternatively, different G-proteins (Blanchet & Lüscher, 2002) or the presence or absence of proteins that mediate the desensitization could be involved. Several studies addressed $GABA_B$ receptor desensitization in cultured neurons. Interestingly, whole-cell recordings of excitatory postsynaptic currents and Kir3 currents in cultured hippocampal neurons reveal that pre- and postsynaptic $GABA_B$ responses exhibit marked differences in their desensitization properties (Wetherington & Lambert, 2002). $GABA_B$-mediated presynaptic inhibition was unaffected by baclofen treatment for up to 48 h, and was desensitized by about one-half after 96 h. In contrast, $GABA_B$-mediated Kir3 currents were desensitized by a similar amount after only 2 h of agonist treatment. Again, it remains unclear whether this difference relates to the receptor or the effector system. A study by Couve et al. provided the first evidence that desensitization of $GABA_B$ receptors is an intrinsic property of the receptor itself (Couve et al., 2002). They showed that direct phosphorylation of a single serine residue (S892) in the cytoplasmic tail of $GABA_{B2}$ by cyclic AMP (cAMP)-dependent protein kinase (PKA) alleviates $GABA_B$ receptor desensitization. $GABA_B$ agonists reduce receptor phosphorylation by reducing cAMP levels and thus enhance desensitization of the receptor, which provides a negative feedback loop. Mechanistically, phosphorylation of S892 enhances the membrane stability of $GABA_B$ receptors. These results are provocative, as they are at odds with current concepts promoting that phosphorylation normally increases GPCR desensitization and internalization. GTPγS binding provides the most direct measure of $GABA_B$ receptor activity, since it assesses the

activation of receptor-associated G-proteins. In cerebellar granule cells GTPγS responses wane in the sustained presence of baclofen, which again provides strong evidence for desensitization being an inherent property of $GABA_B$ receptors (Perroy et al., 2003). This desensitization is dependent on GRK4, a kinase that has been repeatedly implicated in GPCR desensitization. However, the desensitization mechanism is atypical in that it is independent of the kinase activity of GRK4. This again challenges current concepts stating that GRK normally promote GPCR desensitization by phosphorylation of the receptor. Of note, GRK4 is not involved in $GABA_B$ receptor desensitization in most brain regions because of its very restricted spatial expression pattern. The strength and duration of G-protein signaling is attenuated by "Regulator of G-protein Signaling" (RGS) proteins that act as GTPase-activating proteins for G_α subunits. Accordingly, RGS proteins enhance $GABA_B$-dependent desensitization of Kir3.2 currents in transfected HEK293 cells (Mutneja et al., 2005). It is, however, unknown whether RGS proteins modulate native $GABA_B$ responses as well. By the same token, it is generally not known as to what extent desensitization of $GABA_B$ receptors impacts on physiological processes. The fact that desensitization properties differ between neurons and neuronal compartments supports a physiological relevance. For example, non-desensitizing $GABA_B$ receptors may not only respond to local release of GABA from presynaptic terminals, but also mediate a tonic activity in response to ambient GABA concentrations, similar to the high-affinity $GABA_A$ receptors on extrasynaptic membranes (Semyanov et al., 2004).

$GABA_B$ receptors in cultured cortical and hippocampal neurons exhibit little basal endocytosis (Fairfax et al., 2004). Likewise, no agonist-induced endocytosis is observed after a 20 h treatment with baclofen. $GABA_B$ receptor desensitization is therefore not necessarily linked to endocytosis. This is also supported by another study in HEK293 cells (Mutneja et al., 2005). In contrast, $GABA_B$ receptors undergo agonist-induced internalization following desensitization in CHO cells (Gonzalez-Maeso et al., 2003). Similarly, the $GABA_A$ γ2S subunit is reported to enhance $GABA_B$ receptor internalization in response to agonist stimulation (Balasubramanian et al., 2004). Whether $GABA_B$ receptors internalize in response to agonists is therefore controversial and may depend on the cellular context.

5. Tolerance

From a drug discovery point of view, it is important to understand how tolerance to $GABA_B$ agonists develops, and whether tolerance is mechanistically linked to desensitization. Interestingly, some $GABA_B$ responses are more prone to tolerance development than others. For instance, hypothermic and analgesic effects are reduced after repeated administration of baclofen, whereas effects on cAMP production and transient lower esophageal sphincter relaxations are not (Lehmann et al., 2003). The mechanism of tolerance was addressed in a study where tolerance was induced by repeated administration of baclofen to rats once daily for 14 days (Lehmann et al., 2003). Despite the development of complete tolerance to baclofen, $GABA_B$ receptor binding-sites, protein and mRNA levels remained unaltered. Similarly, chronic administration of baclofen did not alter $GABA_{B1}$ and $GABA_{B2}$ mRNA expression in the lumbar spinal cord, despite the development of tolerance to the sedative and antinociceptive effects of the drug (Sands et al., 2003). Taken together, these studies indicate that baclofen induces tolerance through mechanisms other than down-regulation of $GABA_B$ mRNA or protein levels. Whether the development of tolerance involves dephosphorylation events, similarly as proposed for the mechanism of desensitization (Couve et al., 2002), remains to be analyzed.

6. Subcellular localization

In the past, the subcellular distribution of $GABA_B$ receptors has been mainly inferred from synaptosomal release experiments and electrophysiological studies. The availability of $GABA_{B1}$ and $GABA_{B2}$-specific antibodies has now allowed localization studies in defined subcellular compartments using immunogold electron microscopy (Kaupmann et al., 1998; Kulik et al., 2002; Lopez-Bendito et al., 2002; Kulik et al., 2003; Koyrakh et al., 2005). Importantly, several of these studies included $GABA_B$-deficient mice as a control, which confirmed the specificity of the immunolabeling. In general, ultrastructural data corroborate with recombinant studies reporting $GABA_{B1}$ and $GABA_{B2}$ subunit co-localization at the plasma membrane, in agreement with the predominantly heteromeric nature of $GABA_B$ receptors. Immunoreactivity for the $GABA_{B1}$ and $GABA_{B2}$ subunits was observed on presynaptic and, more abundantly, on postsynaptic elements (Fig. 2). Presynaptically, $GABA_B$ subunits were mainly detected in the extrasynaptic membrane and occasionally over the presynaptic membrane specialization of glutamatergic and, to a lesser extent, GABAergic terminals. $GABA_B$ receptors appear to be mostly localized near the active zone, which supports a close link with the release machinery. Presynaptic $GABA_B$ receptors on excitatory terminals in the CA1 region of the hippocampus are proposed to be tonically activated by ambient levels of GABA (Jensen et al., 2003), in agreement with the lack of desensitization seen with these receptors (Wetherington & Lambert, 2002). Similarly, presynaptic $GABA_B$ receptors located on GABAergic terminals impinging onto CA3 interneurons are proposed to be tonically activated by endogenous GABA (Lei & McBain, 2003). An intriguing finding is that $GABA_B$ receptors and their postsynaptic effectors, the Kir3 channels, not only reside in dendritic shafts but also in the extrasynaptic plasma membrane of spines (Drake et al., 1997; Kulik et al., 2003). The spines are small protrusions from dendrites that form the great majority of excitatory synapses, whereas dendritic shafts provide the setting for most inhibitory synapses. The intimate association of $GABA_B$ receptors with glutamatergic synapses at pre- and postsynaptic locations is surprising and suggestive of a tight excitation–inhibition coupling. It is generally assumed that $GABA_B$ receptors on dendritic spines sense GABA spilling over from neighboring GABAergic synapses (Isaacson et al., 1993). This probably requires simultaneous activity of populations of

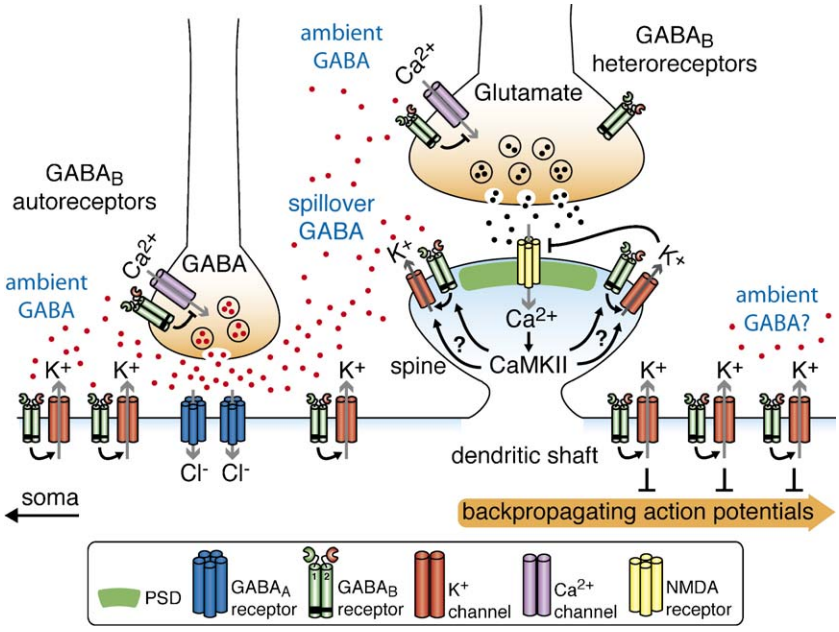

Fig. 2. Localization and physiological roles of $GABA_B$ receptors. $GABA_B$ receptors are located presynaptically, postsynaptically and on extrasynaptic membranes. Presynaptic $GABA_B$ receptors prevent neurotransmitter release by down-regulating the activity of voltage-sensitive Ca^{2+}-channels or by a direct inhibition of the release machinery. $GABA_B$ autoreceptors inhibit the release of GABA, whereas $GABA_B$ heteroreceptors inhibit the release of glutamate and several other neurotransmitters. Some $GABA_B$ heteroreceptors are activated by ambient GABA, others probably by GABA spillover from inhibitory terminals. Postsynaptic $GABA_B$ receptors induce sIPSCs by activating Kir3-type K^+-channels, which hyperpolarizes the membrane, favors voltage-sensitive Mg^{2+} block of NMDA receptors and shunts excitatory currents. $GABA_B$ receptors in spines and dendritic shafts are activated by spillover of GABA from adjacent terminals during population oscillations or during epileptiform activity, which may serve to regulate the excitability of the network and to counteract excess excitation. Dendritic $GABA_B$ receptors inhibit backpropagating action potentials through activation of K^+-channels, which may influence synaptic plasticity processes and action potential generation at the axon hillock. During high-frequency transmission GABA depresses its own release by an action on $GABA_B$ autoreceptors, which permits sufficient NMDA receptor activation for the induction of LTP. In turn, activation of NMDA receptors and CaMKII in dendritic spines enhances the sIPSC mediated by $GABA_B$ receptors and K^+-channels, which is proposed to influence the temporal resolution of synapses. PSD, postsynaptic density.

GABAergic cells as it occurs during population oscillations or epileptic seizures (Scanziani, 2000). The physiological roles of $GABA_B$ receptors in dendritic spines are poorly understood. $GABA_B$ receptors on dendritic spines are highly co-clustered with effector Kir3 channels (Kulik et al., 2006). It is proposed that $GABA_B$-mediated activation of Kir3 channels in the spines counteracts excitatory synaptic currents by hyperpolarization and shunting (Isaacson et al., 1993; Takigawa & Alzheimer, 2003). $GABA_B$ receptors in dendritic spines also modulate metabotropic glutamate receptors (Hirono et al., 2001; Tabata et al., 2004) and reduce synaptic plasticity by favoring the voltage-sensitive Mg^{2+} block of NMDA receptors (Otmakhova & Lisman, 2004). Conversely, activation of NMDA receptors and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) results in the potentiation of sIPSC (Huang et al., 2005). The detailed molecular events underlying the sIPSC potentiation remain unclear, especially whether NMDA receptor activation increases $GABA_B$ receptor or Kir3 channel activity, or both. One possible consequence of the sIPSC potentiation is that late-arriving synaptic events would be less effective because of the increased membrane conductance and hyperpolarization. $GABA_B$ receptors on dendritic shafts show an even distribution and are not necessarily associated with GABAergic boutons. Activation of $GABA_B$ receptors on dendritic shaft delays or inhibits back-propagating action potentials, presumably through increasing the membrane conductance and hyperpolarization (Zilberter et al., 1999; Leung & Peloquin, 2006). This may influence synaptic plasticity processes, action potential generation at the axon hillock and the generation of dendritic Ca^{2+} spikes. It is tempting to speculate that dendritic $GABA_B$ receptors with no association to synapses are also tonically activated by ambient GABA, similarly as observed with high-affinity $GABA_A$ receptors on extrasynaptic membranes (Semyanov et al., 2004).

$GABA_B$ receptors and Kir3 channels are enriched in lipid rafts, as well as the $GABA_B$ receptor associated protein 14-3-3 (Becher et al., 2001; Couve et al., 2001; Koyrakh et al., 2005). It is proposed that $GABA_B$ receptor location to lipid raft domains provides a mechanism for receptor inhibition (Becher et al., 2004). However, it is also proposed that $GABA_{B1a}$, Kir3.1 and Kir3.2 are more abundant in the raft fraction than $GABA_{B1b}$ and Kir3.3, supporting a preferential association of $GABA_{B1}$ isoforms with certain Kir3 channel compositions (Koyrakh et al., 2005). $GABA_B$ receptors may therefore associate with different signaling molecules depending on whether they are present in lipid rafts or not.

7. Summary and outlook

Genetic studies support that the products of the *$GABA_{B1}$* and *$GABA_{B2}$* genes convey all native $GABA_B$ functions. Molecular diversity in the $GABA_B$ system arises primarily from the $GABA_{B1a}$ and $GABA_{B1b}$ subunit isoforms, both of which

combine with the $GABA_{B2}$ subunit to form a heteromeric receptor. Ultrastructural studies show that $GABA_B$ subunits are present in GABAergic and glutamatergic neurons at pre- and postsynaptic sites as well as on extrasynaptic membranes. Thus far, the lack of suitable antibodies has hindered direct visualization of individual $GABA_{B1}$ isoforms in distinct subcellular compartments. Likewise, the lack of selective pharmacological tools has prevented studying the individual contributions of $GABA_{B1a}$ and $GABA_{B1b}$ to pre- and postsynaptic $GABA_B$ functions. Indirect evidence supports a differential segregation of $GABA_{B1a}$ and $GABA_{B1b}$ to pre- and postsynaptic structures (Benke et al., 1999; Billinton et al., 1999; Bischoff et al., 1999). Of note, a differential distribution combined with separate transcriptional control (Steiger et al., 2004) would permit a high degree of plasticity, allowing for dynamically adjustable $GABA_B$ signaling in subcellular compartments. From a pharmaceutical perspective, differences in the signaling between $GABA_{B1}$ isoforms would open up new opportunities for therapeutic interference. It therefore remains a key question whether the molecular composition of $GABA_B$ receptors relates to their subcellular distribution and function. Recent experiments with $GABA_{B1}$ isoform-specific knock-out mice show that this is indeed the case (Vigot et al., in press).

The basic aspects of $GABA_B$ receptor assembly and surface trafficking are now well understood and include the occlusion of an ER retention/retrieval signal in the $GABA_{B1}$ subunit. Several proteins that interact with receptor subunits along the biosynthetic pathway and modulate receptor surface levels were identified. The mechanisms of $GABA_B$ receptor desensitization and internalization are controversial and no conclusive picture has emerged to date. Most studies tend to agree that neuronal $GABA_B$ receptors desensitize in the sustained presence of $GABA_B$ agonists and that desensitization does not involve the same mechanisms as for other GPCR. $GABA_B$ desensitization is not necessarily linked to receptor internalization. Interestingly, $GABA_B$ responses desensitize differently at pre- and postsynaptic sites and in different neurons. Likely, this relates to differences in the associated signaling molecules and possibly to receptor localization and subunit composition. An emerging topic is the crosstalk of $GABA_B$ receptors with metabotropic and ionotropic glutamatergic receptors in dendritic spines, a location where $GABA_B$ receptors and Kir3 effector channels were found to be unusually abundant.

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