Structural Basis of $GABA_B$ Receptor Regulation and Signaling

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Contents

Abstract $GABA_B$ receptors (GBRs), the G protein-coupled receptors for the inhibitory neurotransmitter γ-aminobutyric acid (GABA), activate Go/i-type G proteins that regulate adenylyl cyclase, Ca^{2+} channels, and K^+ channels. GBR signaling to enzymes and ion channels influences neuronal activity, plasticity processes, and network activity throughout the brain. GBRs are obligatory heterodimers composed of GB1a or GB1b subunits with a GB2 subunit. Heterodimeric GB1a/2 and GB1b/2 receptors represent functional units that associate in a modular fashion with regulatory, trafficking, and effector proteins to generate receptors with distinct physiological functions. This review summarizes current knowledge on the structure, organization, and functions of multi-protein GBR complexes.

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1 Heterodimers Are the Minimal Functional Receptor Units

The GB1a and GB1b subunit isoforms were cloned in 1997 using a radioligand binding approach (Kaupmann et al. 1997). This showed that GBRs belong to class C GPCRs, which include metabotropic glutamate receptors, Ca^{2+}-sensing receptors, and taste receptors (Kaupmann et al. 1997). As for other class C GPCRs, GB1 subunits contain a large extracellular venus fly trap domain (VFTD), the typical heptahelical transmembrane domain (7TMD), and an intracellular C-terminal domain (Fig. 1). When expressed in heterologous cells, GB1 subunits exhibited ~tenfold lower affinity for GABA than native GBRs (Kaupmann et al. 1997, 1998; White et al. 1998). In addition, GB1 subunits failed to exit the endoplasmic reticulum (Couve et al. 1998) and to efficiently inhibit adenylyl cyclase or activate Kir3 channels (Kaupmann et al. 1997). This showed that GB1 subunits do not form functional receptors by themselves. Soon after cloning of GB1 subunits, cDNA homology searches and yeast-two-hybrid screens identified the sequence-related GB2 subunit (Kaupmann et al. 1998; Kuner et al. 1999; Ng et al. 1999; White et al. 1998). GB2 was again non-functional when expressed in heterologous cells (Galvez et al. 2001). In situ hybridization studies showed that neurons generally co-express GB1 and GB2 transcripts, which indicated that GB1 and GB2 subunits might function together in a heterodimeric receptor. Co-expression of GB1 with GB2 subunits indeed generated receptors with tenfold higher affinity for GABA that also efficiently signaled to neuronal effectors, including adenylyl cyclase, Kir3 channels, and P/Q/N-type Ca^{2+} channels (Kaupmann et al. 1998; Kuner et al. 1999; Ng et al. 1999; White et al. 1998). This finding represented the earliest demonstration of an obligatory heterodimeric G protein-coupled receptor (Marshall et al. 1999). The GB1a and GB1b subunit isoforms derive from the same gene by differential promoter usage and exhibit distinct expression patterns in the central nervous system (Bischoff et al. 1999). Structurally, GB1a differs from GB1b by the presence of two sushi domains in the N-terminal domain (SD1, SD2) (Blein et al. 2004) (Fig. 1). The N-terminal SD1 has an intrinsically disordered structure, while SD2 is more compactly folded. The SDs function as axonal trafficking signals (Biermann et al. 2010) and stabilize the receptor at the cell surface (Hannan et al. 2012). Accordingly, axons predominantly express GB1a/2 receptors, while the somatodendritic compartment expresses GB1b/2 receptors. However, GB1a/2 receptors are also present in the dendrites but excluded from the spine heads, in contrast to GB1b/2 receptors (Biermann et al. 2010; Dinamarca et al. 2019; Vigot et al. 2006).

GBRs have evolved quality control signals that prevent unfolded or unassembled subunits from exiting the endoplasmic reticulum (ER) and the Golgi apparatus. The

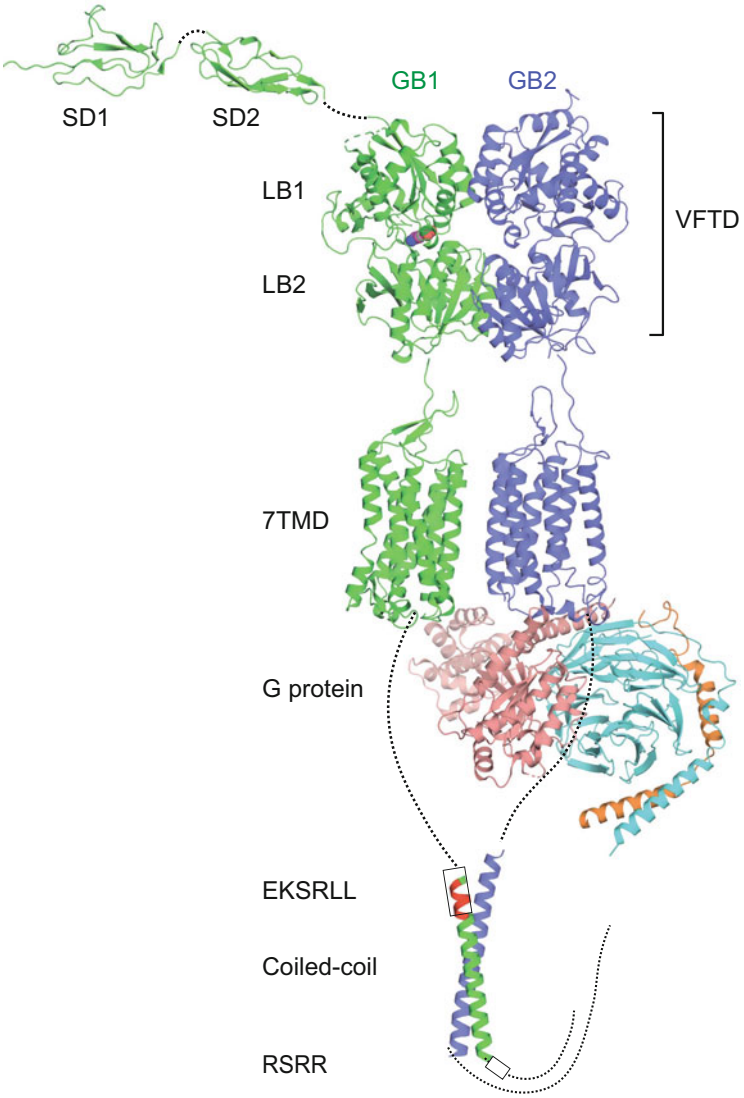

Fig. 1 Structural model of the GBR heterodimer. The model is based on the published structures of SD1 [PDB ID: 6HKC (Rice et al. 2019)], SD2 [1SRZ (Blein et al. 2004)], baclofen-bound VFTDs [4MS4 (Geng et al. 2013)], active and inactive mGlu5 [6N51 and 6N52 (Koehl et al. 2019)], the coiled-coil domain of GBRs [4PAS (Burmakina et al. 2014)] and the heterotrimeric G protein complex [3SN6 (Rasmussen et al. 2011)]. GB1 and GB2 are colored in green and slate, respectively. The active and inactive conformations of the $7TMD_{mGlu5}$ were used in GB1 and GB2, respectively. The boxes in the C-terminal domain of GB1 indicate the retention motifs RSRR and EKSRLL that control heterodimer assembly during biosynthesis

intracellular C-terminal domain of GB1 subunits encodes the ER retention signal RSRR, which is located distal to a coiled-coil heterodimerization domain (Margeta-Mitrovic et al. 2000; Pagano et al. 2001) (Fig. 1). Prenylated rab acceptor family 2 (PRAF2) protein, an ER-resident molecule, binds to the ER retention signal and prevents exit of the GB1 subunit from the ER (Doly et al. 2016). Coiled-coil heteromerization of GB1 with GB2 subunits competitively displaces PRAF2 from its binding motif and enables forward trafficking of the GB1/2 heterodimer in the biosynthetic pathway. The coat protein complex I (COPI) also binds to the ER retention signal and shuttles unassembled GB1 subunits from the cis-Golgi back to the ER (Brock et al. 2005). An additional signal within the coiled-coil domain of GB1, the di-leucine motif EKSRLL (Fig. 1), controls release of receptors from the trans-Golgi network (Restituito et al. 2005). Msec7-1, a guanine-exchange factor protein of the ARF family of GTPases, binds to this di-leucine motif and prevents exit of unassembled GB1 subunits from the Golgi apparatus. Structural data show that heterodimerization of GB1 with GB2 subunits occludes the di-leucine signal and prevents Msec7-1 from binding (Burmakina et al. 2014).

At high cell surface density, GB1/2 heterodimers assemble by random collision into higher-order oligomers of two or more heterodimers (Calebiro et al. 2013; Comps-Agrar et al. 2011; Maurel et al. 2008; Schwenk et al. 2010; Stewart et al. 2018). It appears that in higher-order oligomers GB1 subunits arrange in a line via the opposite sides of their 7TMDs, while GB2 subunits are on the side (Xue et al. 2019). Mutation of either E380 + L382, T410 + E412, or E413 in the VFTD of rat GB1a ($VFTD_{GB1a}$) disrupts the formation of higher-order oligomers (Comps-Agrar et al. 2011; Stewart et al. 2018). Molecular modeling indicates that two G proteins can couple to one GBR tetramer (Xue et al. 2019). Interestingly, however, higher-order oligomerization limits the capacity of GBRs to activate G proteins, presumably because only one of the agonist binding sites in the two neighboring GB1 subunits of a GBR tetramer can be occupied (Stewart et al. 2018). It is unknown whether suppression of G protein signaling in higher-order oligomers is of regulatory significance or not.

2 Signal Transduction in the Receptor Heterodimer

GB1 and GB2 subunits fulfill distinct functions in the receptor heterodimer. Only GB1 contains a GABA binding site (Galvez et al. 1999), whereas GB2 couples to the G protein (Duthey et al. 2002; Havlickova et al. 2002). GB2 additionally allosterically increases GABA affinity at GB1 (Kaupmann et al. 1998; White et al. 1998). After binding of GABA at GB1, multiple allosteric interactions between subunit domains are necessary to activate the G protein at GB2. X-ray structures of the extracellular dimerization interface are now available (Geng et al. 2012, 2013). The $VFTD_{GB1}$/$VFTD_{GB2}$ dimer structure shows that GB1 and GB2 subunits interact sideways, facing opposite directions (Geng et al. 2013) (Fig. 2). Each VFTD has a bi-lobed structure, where lobe 1 (LB1) is positioned more distant from the plasma

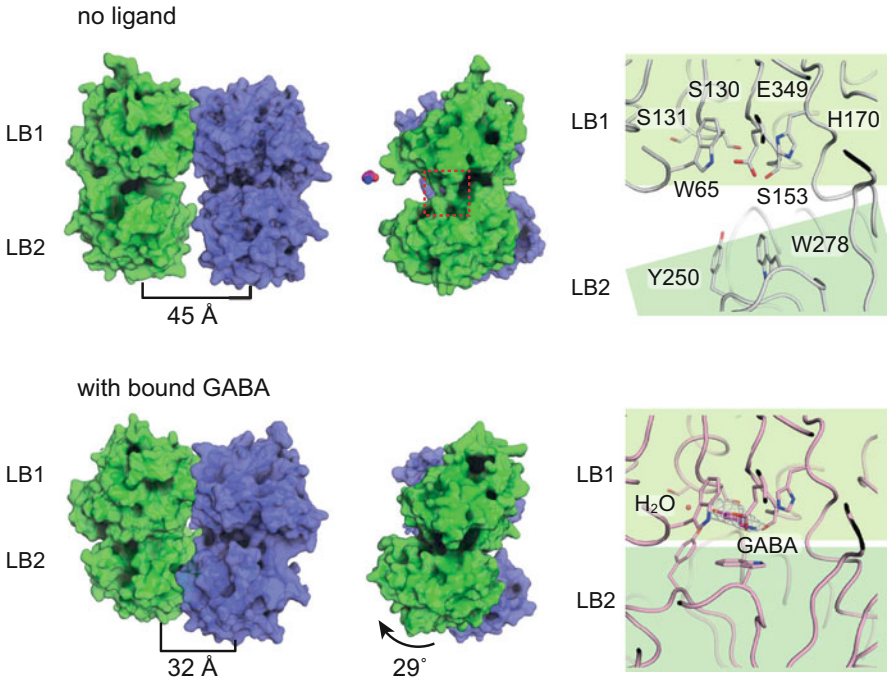

Fig. 2 Conformational changes in VFTDs during receptor activation. Structures of the VFTDs in the absence of a ligand [PDB ID: 4MQE, top] and with GABA bound to $VFTD_{GB1}$ [4MS3, bottom (Geng et al. 2013)] are shown. Upon GABA binding, LB2 of $VFTD_{GB1}$ (green) rotates by 29° and moves toward $VFTD_{GB2}$ (slate) by ~10 Å. In contrast, LB2 of $VFTD_{GB2}$ rotates only by 9° upon activation. Amino acid residues in $VFTD_{GB1}$ involved in the binding of GABA are shown on the right, with numbering of residues according to human GB1b (Geng et al. 2013). Y250, W278, and S131 (water mediated contact) on LB2 interact with GABA and subsequently close the interface between the LB domains. The 2F(o)-F(c) electron density map of GABA is shown as mesh at $\sigma = 1.5$ (right bottom)

membrane than LB2. A peptide hinge connects LB1 and LB2, enabling LB2 to move in relation to LB1. In the heterodimer, $VFTD_{GB1}$ and $VFTD_{GB2}$ bind to each other via LB1. The LB1/LB1 interaction is stabilized by multiple hydrophobic contacts (Y113/Y117 in GB1b; Y118/W149 in GB2, amino acid numbering refers to human sequences in the remainder of the manuscript), salt bridges (R141 in GB1b, D109 in GB2), hydrogen bonds (E138 in GB1b, N110 in GB2), and multiple van der Waals contacts (Geng et al. 2013). The hydrophobic patch is located at the center of the interface and flanked by sites forming hydrogen bonds and water-mediated contacts.

Mutagenesis and X-ray crystallography studies show that agonists and antagonists bind to a large crevice between LB1 and LB2 in $VFTD_{GB1}$ (Galvez et al. 1999; Geng et al. 2013). Agonists and antagonists form multiple interactions with residues in LB1, including hydrogen bonds with S130, S153, H170, and E349, van der Waals contacts with W65, and water-mediated contacts with S131 (GB1b numbering)

(Geng et al. 2013) (Fig. 2). Agonists additionally bind to Y250 and W278 in LB2. The antagonists CGP54626 and SCH50911 also bind to W278 in LB2, which increases antagonist-binding affinity (Geng et al. 2013). The large substituents at either side of antagonists physically prevent $VFTD_{GB1}$ closure, which stabilizes $VFTD_{GB1}$ in an open conformation (Geng et al. 2013). Conversely, agonists induce $VFTD_{GB1}$ closure (Geng et al. 2012; Kniazeff et al. 2004). Mutations that stabilize $VFTD_{GB1}$ closure therefore lead to constitutive activity. Upon agonist binding, LB2 in $VFTD_{GB1}$ rotates 29° about a nearly horizontal axis, bringing LB2 close to LB1 and closing $VFTD_{GB1}$ (Fig. 2). This rotation additionally moves $VFTD_{GB1}$ closer to LB2 of $VFTD_{GB2}$ that remains in a constitutively open conformation stabilized by hydrogen bonds between LB1 and LB2 (Geng et al. 2012). LB2 of $VFTD_{GB2}$ twists by ~9° about a nearly vertical axis, moving it toward LB2 of $VFTD_{GB1}$. As a result, a new LB2/LB2 interface forms that stabilizes $VFTD_{GB1}$ in the closed conformation and increases agonist affinity. The LB2/LB2 interface is essential for receptor activation, as disruption of the interface by insertion of a glycan wedge precludes receptor activation (Rondard et al. 2008). Conversely, a covalent disulfide bridge linking the LB2 lobes locks the receptor in a constitutively active state (Geng et al. 2013). While agonist binding promotes $VFTD_{GB1}$/$VFTD_{GB2}$ interaction, it simultaneously causes a spatial reorientation of $7TMD_{GB1}$ and $7TMD_{GB2}$ that enables activation of the G protein at $7TMD_{GB2}$ (Matsushita et al. 2010; Monnier et al. 2011). Allosteric activation of $7TMD_{GB2}$ occurs in *cis* and in *trans* via $VFTD_{GB2}$ and $7TMD_{GB1}$, respectively (Monnier et al. 2011). Receptor activation disrupts an ionic lock at the intracellular side of $7TMD_{GB2}$ (Binet et al. 2007). The ionic lock is formed by a salt bridge between D688 in TM6 and K574 in TM3, which prevents outward movement of TM6 and stabilizes the inactive closed conformation of the $7TMD_{GB2}$. Disruption of the ionic lock by mutation allosterically increases agonist affinity. Recent studies show that crosslinking of the TM6-TM6 interaction between GB1 and GB2 is sufficient for receptor activation and leads to constitutive activity (Xue et al. 2019). Interestingly, GBRs also exhibit constitutive activity in the absence of agonists (Galvez et al. 2001; Grunewald et al. 2002). This suggests that GBRs exhibit high intrinsic conformational flexibility and spontaneously oscillate between inactive and active states, similar as shown for the isolated VFTDs of metabotropic glutamate receptor 2 (Olofsson et al. 2014).

Almost all known allosteric modulators of GBRs bind to $7TMD_{GB2}$ (Binet et al. 2004; Dupuis et al. 2006; Sun et al. 2016). The only exception is Ca^{2+}, which binds to S269 in LB1 of GB1a (S153 in GB1b) and thereby increases affinity for GABA (Galvez et al. 2000; Wise et al. 1999) (Fig. 2). Positive allosteric modulators (PAMs) at GBRs generally increase agonist potency and efficacy (Urwyler et al. 2005). Some PAMs also have agonistic properties and activate the receptor in the absence of orthosteric agonists, presumably by stabilizing the active conformation of $7TMD_{GB2}$. The negative allosteric modulator (NAM) CLH304 has inverse agonist properties, suppressing basal activity as well as agonist-induced receptor activation, likely by preventing $7TMD_{GB2}$ from reaching the active conformation (Chen et al. 2014; Sun et al. 2016). The binding sites of allosteric modulators in $7TMD_{GB2}$ are unknown. Crystal structures of other class C G protein-coupled receptors suggest

that allosteric modulators enter a cavity located between transmembrane domains 3, 5, 6, and 7 (Christopher et al. 2015; Dore et al. 2014; Gregory et al. 2011; Wu et al. 2014). A GB2 homology model predicts a hydrophobic binding pocket in $7TMD_{GB2}$ and identified potential amino acid residues involved in binding of allosteric modulators (Freyd et al. 2017).

3 Auxiliary KCTD Subunits

Several observations pointed to native GBRs being composed of more than just a GB1 and a GB2 subunit. For example, GBR complexes isolated from brain tissue had molecular masses of 0.6–1.1 MDa, while the heterodimer only accounts for 240 kDa (Schwenk et al. 2010). Moreover, the kinetic properties of native GBR responses varied and differed from those of GB1/2 heterodimers expressed in heterologous cells (Turecek et al. 2014). Quantitative proteomic approaches identified approximately 30 proteins that interact with GB1 or GB2 in the brain (Dinamarca et al. 2019; Schwenk et al. 2010, 2016; Turecek et al. 2014). These proteins provide a molecular basis to explain the functional diversity of native GBRs. Known interactions between components of GBR complexes have been summarized recently (Fritzius and Bettler 2020).

Abundant GBR-interacting proteins are the K^+ channel tetramerization domain (KCTD) proteins KCTD8, KCTD12, KCTD12b, and KCTD16 (herein collectively referred to as the KCTDs) (Schwenk et al. 2010). The KCTDs are part of a larger family of KCTD proteins comprising 26 members with sequence similarity to the cytoplasmic tetramerization (T1, also known as BTB or POZ) domain of voltage-gated K^+ channels (Correale et al. 2013; Zheng et al. 2019). The KCTDs are composed of the N-terminal T1 domain and a H1 domain, with both isolated domains capable of forming oligomers (Correale et al. 2013; Fritzius et al. 2017). KCTD8 and KCTD16 additionally encode a C-terminal H2 domain that scaffolds effector channels and other receptor-associated proteins (see below). Structural studies demonstrate that $T1_{KCTD12}$ and $T1_{KCTD16}$ form homopentamers (Pinkas et al. 2017; Smaldone et al. 2016; Zheng et al. 2019; Zuo et al. 2019) (Figs. 3a and 5). The $T1_{KCTD16}$ pentamer is open, with a gap of 8–16 Å at its narrowest and widest points. Since one $T1_{KCTD16}$ monomer in the pentamer occupies 25 Å, the gap in the pentamer is too small to accommodate a sixth $T1_{KCTD16}$ monomer. Multiple electrostatic interactions and nonpolar associations stabilize adjacent $T1_{KCTD16}$ domains. Most of the conserved amino acid residues are involved in $T1_{KCTD16}$ interactions, supporting that all four KCTDs assemble as pentamers. Co-crystallization of $T1_{KCTD16}$ with a C-terminal GB2 peptide shows that the $T1_{KCTD16}$ pentamer wraps around the peptide (Fig. 3a, b). The GB2 peptide loops inside the central opening of the pentamer, entering and leaving the pentamer at its N-terminal surface. The apex of the GB2 peptide loop forms a short helix that contains the Y903 residue critical for KCTD binding (Correale et al. 2013; Schwenk et al. 2010; Zheng et al. 2019) (Fig. 3a). X-ray crystallography reveals that Y903 is

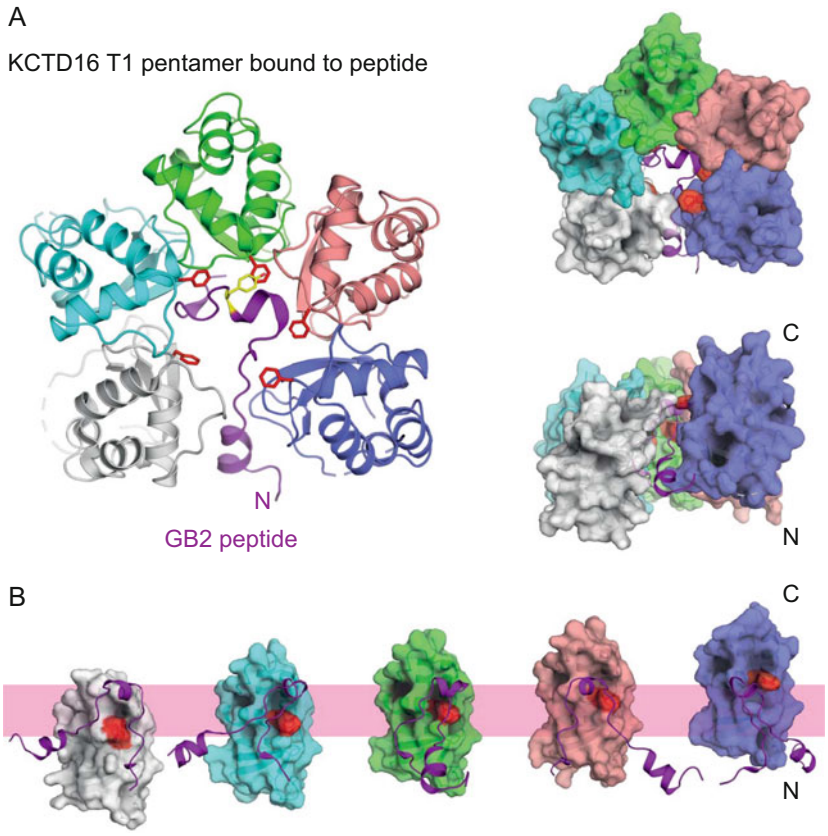

Fig. 3 Binding of the GB2 C-terminus to the $T1_{KCTD16}$ pentamer. (**a**) Structure of a GB2 C-terminal peptide bound to the $T1_{KCTD16}$ pentamer [PDB ID: 6M8R (Zheng et al. 2019)]. The F80 (red) and Y903 (yellow) residues in $T1_{KCTD16}$ and GB2, respectively, are highlighted. $T1_{KCTD16}$ domains form an open pentamer with C6 symmetry. A twist of the ring prevents the sixth subunit from being inserted. Upon binding to the GB2 C-terminal peptide, the open pentamer contracts by roughly 4–5 Å and creates a tight channel for the peptide (right). The orientation of the complex is indicated by the N- and C-terminus of KCTD16. (**b**) The twisted ring structure enables each $T1_{KCTD16}$ subunit to form a distinct binding interface with the peptide. A cross-section of the pentamer shows the interaction of each of the five $T1_{KCTD16}$ domains with the GB2 peptide. The F80 residues (red) and the $T1_{KCTD16}$ domains are aligned vertically according to the position of GB2 peptide

located in the middle of an extensive interaction interface (Zuo et al. 2019). The interaction takes place off center in the central pore of the pentamer, opposite of the gap. In each $T1_{KCTD16}$ domain of the pentamer, the F80 residue protrudes into the central pore. A slight offset due to a tilt of each $T1_{KCTD16}$ monomer forms a spiraling ladder of F80 residues in the inner wall of the pentamer (Fig. 3b). This arrangement allows F80 residues to bind many side chains in a GB2 peptide of 25 amino acid residues (Zheng et al. 2019). Consistent with the X-ray data, the F80A mutation

completely abrogates KCTD16 binding to GBRs (Zuo et al. 2019). Interestingly, binding of GB2 results in a compaction of the $T1_{KCTD16}$ pentamer (Fig. 3a).

Reverse affinity purification experiments using KCTD-specific antibodies revealed that KCTD8, KCTD12, KCTD12b, and KCTD16 not only bind to GB2 but also to the Gβγ subunits of the G protein (Turecek et al. 2014; Zheng et al. 2019). Co-crystallization studies show that each $H1_{KCTD12}$ pentamer binds five Gβγ subunits in a near perfect C5 rotational symmetry (Zheng et al. 2019) (Fig. 4). The five Gβγ molecules form a tightly packed outer ring in which every Gβ subunit directly contacts neighboring Gβ subunits as well as two adjacent H1 domains of the pentamer. $H1_{KCTD12}$ folds into a β sheet made up by five antiparallel β strands (β1-5) interspersed with two short α helices (α1 and α2). The amino acids at and around the loops between β1/β2 as well as β3/α2 bind to an acidic patch at the top of the Gβ propeller (interface I) and a groove between the N-terminal helix and the β propeller of Gβ (interface II) (Fig. 4). In $H1_{KCTD12}$, R232 (contacting interface I on Gβ) and R257 (contacting interface II on Gβ) are particularly important for the interaction, as mutation of either residue completely abolishes Gβγ binding and modulation of G protein signaling by KCTD12. The Gγ subunit is located peripherally and does not interact with the H1 domain. However, Gγ allows anchoring of the complex at the plasma membrane (Figs. 4 and 5). When incubating $KCTD12_{H1}$ with a substoichiometric amount of Gβγ, only full 5/5 complexes and free KCTD12 were observed, with no evidence of partial oligomers (Zheng et al. 2019). This suggests that binding of $H1_{KCTD12}$ to Gβγ is highly cooperative. Supported by 3D reconstructions of electron microscopy images of the full-length KCTD12 protein in complex with Gβγ (Zheng et al. 2019), the picture of a large multi-protein complex emerges, in which KCTDs simultaneously bind via their T1 and H1 domains GB2 and Gβγ subunits, respectively (Fig. 5). Of note, Gβγ binding to the $H1_{KCTD12}$ pentamer partially occludes the Gα binding-site on the surface of Gβγ, indicating that the trimeric G protein does not assemble with KCTD12 into a pentameric complex. This contrasts earlier biochemical findings that support that GBRs and KCTDs form a complex with the heterotrimeric G protein (Turecek et al. 2014). Co-crystallization of the H1 domain with Gβγ may therefore favor a structure that differs from the structure of the full-length KCTD protein assembled with receptor and the heterotrimeric G protein.

Dual binding of the KCTDs to the receptor and the G protein enables KCTDs to regulate the kinetics of receptor signaling (Fritzius et al. 2017; Schwenk et al. 2010; Seddik et al. 2012; Turecek et al. 2014). Pre-assembly of the G protein via the KCTDs at the receptor significantly accelerates G protein signaling, most likely by overcoming slow diffusion-limited association of the G protein with the receptor (Turecek et al. 2014). When studying GBR-mediated K^+ current responses, KCTDs shorten both the rise time and the delay between agonist binding and the onset of K^+ currents. The KCTDs are therefore responsible for the fast kinetics observed with GBR-induced current responses in neurons (Schwenk et al. 2010; Turecek et al. 2014). While all KCTDs accelerate GBR signaling, selectively KCTD12 and KCTD12b induce a rapid desensitization of GBR-mediated K^+ currents (Schwenk et al. 2010; Seddik et al. 2012) through an activity-dependent uncoupling of Gβγ

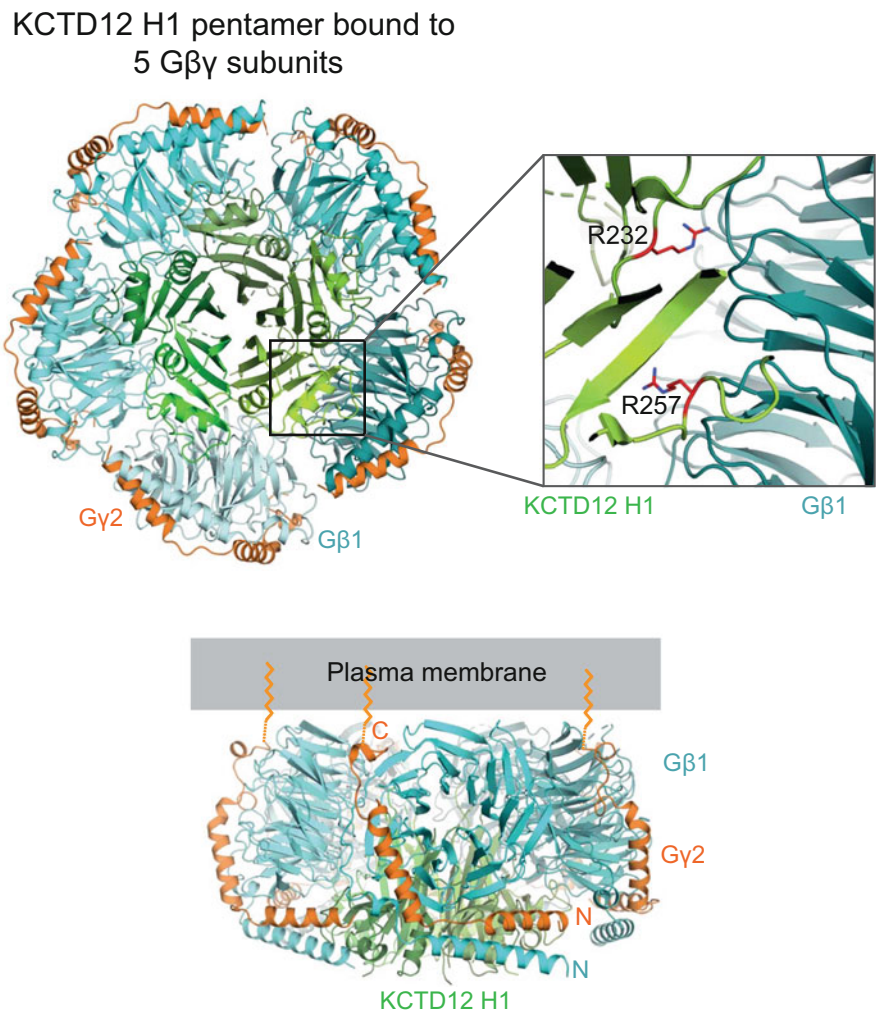

Fig. 4 The $H1_{KCTD12}$/Gβγ complex. Top: $H1_{KCTD12}$ pentamers and five Gβ1γ2 subunits form together a complex with C5 symmetry [PDB ID: 6M8S, (Zheng et al. 2019)]. Each of the five Gβ1γ2 subunits binds two $H1_{KCTD12}$ subunits. The cutout shows R232 (interface I) and R257 (interface II) that are crucial for Gβ1 recognition. Bottom: Due to lipidation of Gβγ subunits, the complex is expected to be tethered to the plasma membrane

from effector channels (Turecek et al. 2014; Zheng et al. 2019). Some neuronal populations simultaneously express multiple KCTDs, raising the possibility that the KCTDs form hetero-oligomers. Indeed, in heterologous cells, KCTD8, KCTD12, and KCTD16 form hetero-oligomers in all possible combinations (Balasco et al. 2019; Fritzius et al. 2017). Association of KCTD12/16 hetero-oligomers with GBRs in hippocampal pyramidal cells confers unique kinetic properties to GBR-induced K^+ currents, showing that hetero-oligomers increase the kinetic repertoire of GBR

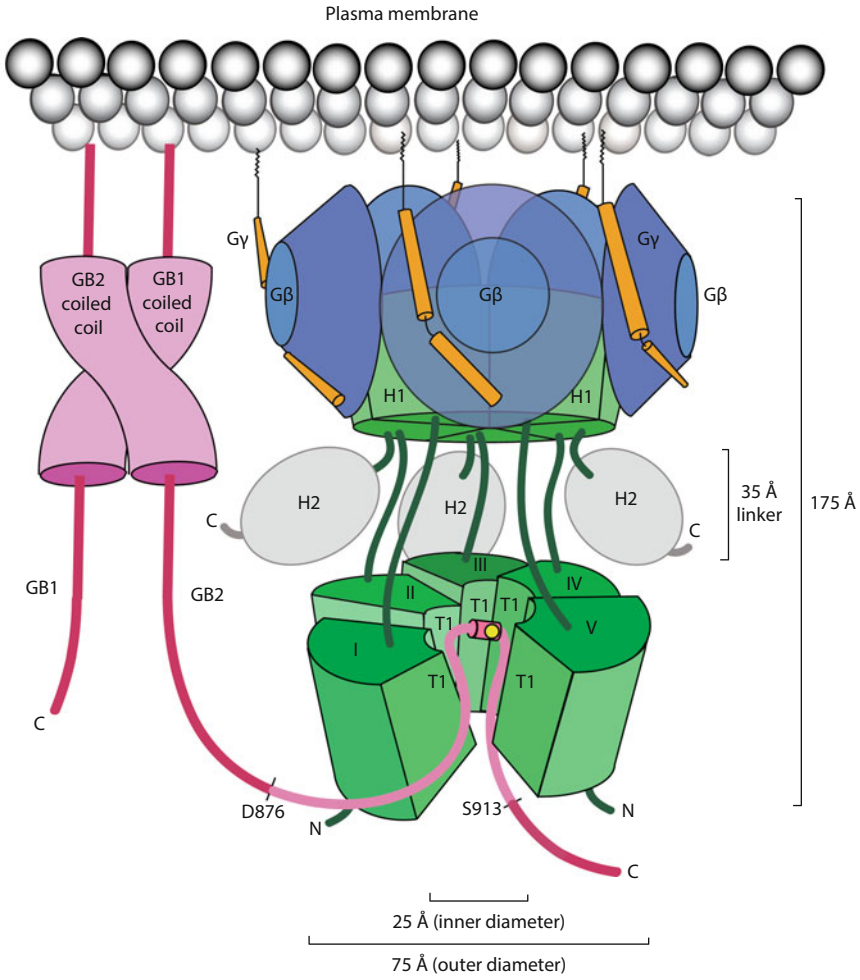

Fig. 5 Scheme of the multi-protein GBR/KCTD12/G protein signaling complex. The intracellular part of the GBR (dark pink), a pentamer of KCTD12 proteins (green), Gβ (blue), and Gγ (orange) subunits are depicted. The N-terminal T1 domain of the KCTDs forms an open pentamer that interacts with the cytoplasmic tail of GB2 (the GB2 peptide, containing the amino acids D888 to S913, co-crystallized with the $T1_{KCTD16}$ is highlighted in bright pink). This part of GB2 loops inside the central opening of the T1 pentamer, entering and leaving it at its N-terminal surface. The amino acid Y903 (yellow circle) at the apex of the GB2 loop is critical for KCTD binding. A slight offset due to a tilt of each $T1_{KCTD16}$ monomer allows the pentamer to bind a large number of amino acid side chains within the cytoplasmic tail of GB2. A short linker (35 Å) connects the N-terminal T1 domain with the C-terminal H1 domain of KCTDs, which binds to the Gβγ heterodimer of the G protein. The scheme depicts the closed $H1_{KCTD12}$ pentamer bound to five copies of Gβγ. Anchoring of the Gγ subunit to the phospholipid bilayer tethers KCTDs to the plasma membrane. The expected location of the C-terminal H2 domain present in KCTD16 and KCTD8 is indicated for three of the KCTDs in the pentamer. Distances are derived from three-dimensional negative-stain electron microscopy reconstructions (Zheng et al. 2019)

signaling (Fritzius et al. 2017). Of note, the KCTDs exert little influence on allosteric and orthosteric binding sites of GBRs (Rajalu et al. 2015).

Reverse-affinity purification experiments support that the KCTDs do not bind to other GPCRs (Schwenk et al. 2016; Turecek et al. 2014). The KCTDs are non-obligatory GBR components, which, however, are expressed by most neurons (and some glial cells) in the vertebrate brain (Metz et al. 2011). Since the KCTDs stably associate with the receptor and control receptor kinetics and surface expression (Ivankova et al. 2013), they should be viewed as auxiliary receptor subunits.

4 SD-Interacting Proteins

Proteomic studies showed that the β-amyloid precursor protein (APP), the adherence junction-associated protein 1 (AJAP-1), and the PILRα-associated neural protein (PIANP) form three distinct complexes with GB1a/2 receptors (Dinamarca et al. 2019; Schwenk et al. 2016). NMR studies identified sequence-related epitopes in the extracellular domains of APP, AJAP-1, and PIANP that bind with nanomolar affinities to the N-terminal SD1 of GB1a, with a rank order of affinities AJAP-1 > PIANP >> APP (Dinamarca et al. 2019). APP is best known as the source of β-amyloid (Aβ) peptides in Alzheimer's disease. Electrophysiological and biochemical experiments showed that binding of APP to GB1a is necessary for vesicular trafficking of GBRs to axon terminals (Dinamarca et al. 2019), consistent with the proposed role of the SDs in axonal trafficking (Biermann et al. 2010). Proteomic data show that APP associates with calsyntenins and c-Jun N-terminal kinase-interacting proteins (JIPs) that link the APP/GBR complex in cargo vesicles to the axonal kinesin-1 motor. Of potential relevance for Alzheimer's disease, complex formation with GBRs stabilizes APP at the cell surface and reduces proteolysis of APP to Aβ (Dinamarca et al. 2019). A related study showed that binding of the soluble form of APP (sAPP) to the SD1 of GB1a inhibits neurotransmitter release, synaptic transmission and spontaneous neuronal activity (Rice et al. 2019). The fact that a GBR antagonist disinhibits sAPP-inhibited neurotransmitter release supports that sAPP acts as a GBR agonist or positive allosteric modulator. However, it was also reported that sAPP has no functional effects on GBR signaling in heterologous cells (Dinamarca et al. 2019). Therefore, additional studies need to confirm sAPP effects on GBR signaling. AJAP-1 and PIANP, the two other proteins binding to the SD of GB1a, do not play a role in axonal trafficking of GBRs (Dinamarca et al. 2019). These proteins localize to adherens junctions that stabilize cell-cell interactions (Winkler et al. 2019; Yamada and Nelson 2007) and may be important for anchoring GB1a/2 receptors at presynaptic sites, either in *cis* or through trans-synaptic interactions. In support of this hypothesis, PIANP knock-out mice exhibit a deficit in GBR-mediated inhibition of glutamate release in the hippocampus (Winkler et al. 2019).

Amyloid-like protein 2 (APLP2) and integral membrane protein 2B (ITM2B) and ITM2C are additional transmembrane proteins that selectively co-purify with GB1a/

2 receptors (Dinamarca et al. 2019; Schwenk et al. 2016). These proteins associate with APP and are secondary interactors of GBRs. GBRs can therefore assemble with multi-protein APP complexes into super-complexes (complexes of complexes).

5 Effector Channels

The best-studied GBR functions in the central nervous system are the gating of voltage-sensitive Ca^{2+} (Ca_v) channels and inwardly rectifying Kir3-type K^+ channels by the Gβγ subunits of the activated G protein (Gassmann and Bettler 2012). GBRs inhibit N- and P/Q-type Ca_v channels, which suppress neurotransmitter release at most synapses in the brain. GBR activation of Kir3 channels hyperpolarizes the membrane, shunts postsynaptic currents in the dendrites, and inhibits neuronal firing. The α1B, α2, δ1, and δ2 subunits of N-type Ca_V channels co-purify with the GB1, GB2, and KCTD16 subunits, supporting that these channels bind to GBRs via KCTD16 (Schwenk et al. 2016). Association of GBR with N-type Ca_V channels directly links the receptor to the presynaptic release machinery. Proteomic work did not support a physical association of native GBRs with Kir3 channels (Schwenk et al. 2016), in contrast to earlier studies in heterologous expression systems (Ciruela et al. 2010; David et al. 2006; Fowler et al. 2007). It is possible that proteomic approaches miss weak interactions of Kir3 channels with GBRs. Alternatively, overexpression of two membrane proteins in heterologous cells may lead to artificial aggregates detected in BRET and immunoprecipitation experiments. Proteomic work additionally identified novel effector channels of GBRs, such as the transient receptor potential vanilloid 1 (TRPV1) (Hanack et al. 2015) and HCN2 channels (Schwenk et al. 2016). Sensitization of TRPV1 channels is central to the initiation of pathological forms of pain. TRPV1 assembles in a complex with GB1 (Hanack et al. 2015). Since agonist activity at GB1 reverts the sensitized state of TRPV1 channels, it may be possible to exploit the TRPV1/GB1 complex for anti-pain therapy. HCN1 and HCN2, like N-type Ca_v channels, appear to associate with GBRs through KCTD16 (Schwenk et al. 2016). HCN channels are widely expressed in the heart and the central nervous system, where they are involved in the generation of rhythmic activity (Biel et al. 2009). GBRs activate HCN currents in dopaminergic neurons of the ventral tegmental area and thereby shorten the duration of inhibitory postsynaptic potentials (Schwenk et al. 2016). The mechanism of GBR-induced HCN channel activation is unknown but may include (1) membrane hyperpolarization via Kir3 channels, (2) allosteric gating of HCN channels by conformational changes in the receptor, and/or (3) dynamic interactions of HCN channels with G protein subunits or second messengers.

6 Additional Receptor-Associated Proteins

Additional components of native GBR signaling complexes are calnexin, reticulocalbin-2, inactive dipeptidyl-peptidases 6/10, 14-3-3 proteins, synaptotagmin-11, and neuroligin-3 (Schwenk et al. 2016). The anatomically and temporally restricted expression of these proteins in the brain limits the set of available receptor constituents in individual cells and further supports a modular GBR architecture. For some of these receptor components binding sites on GB1, GB2, or the KCTDs have been identified (Fritzius and Bettler 2020). Yeast-two-hybrid screens identified several additional proteins that potentially interact with GB1 or GB2 (Pin and Bettler 2016). These proteins may represent low-abundance or transiently interacting GBR components that escaped detection in proteomic approaches.

7 Concluding Remarks

During the past decade, numerous structural and biophysical studies have greatly improved our understanding of the sequence of allosteric events involved in the activation of heterodimeric GBRs. However, the structures of the full-length heterodimeric GBR at atomic resolution in its active and inactive state, with and without bound G protein or allosteric modulators, are still missing. Cryo-electron microscopy appears to be a promising approach to obtain such high-resolution structural information, which is necessary to validate and extend current concepts. The functional relevance of higher-order GBR complexes is still unclear and needs to be addressed in native tissue. The recognition that GBR heterodimers interact with an inventory of ~30 proteins to form a variety of multi-protein complexes with distinct kinetic properties, localizations, and functions represents a departure from earlier concepts based on receptor protomers working in isolation. For some GBR interacting proteins (KCTDs, APP, HCN channels), we have identified functional effects and/or obtained high-resolution structures in association with the receptor. However, we still lack functional and structural information for most of the receptor components identified in proteomic approaches. Furthermore, much effort needs to be devoted to the study of the structural dynamics in GBR complexes during physiological processes. Understanding the structure and function of identified GBR complexes in the brain hopefully will help to identify promising molecular targets for therapeutic intervention.

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