MODULATION OF $GABA_B$ RECEPTOR SIGNALING BY ASSOCIATED PROTEINS AND PHOSPHORYLATION

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I. SUMMARY

$GABA_B$ receptors are the G protein-coupled receptors (GPCRs) for γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the central nervous system. They are membrane receptors with a seven membrane-spanning domain and are composed of the principal subunits $GABA_{B1}$ and $GABA_{B2}$. Activated $GABA_B$ receptors regulate G protein-coupled inwardly rectifying K^+ channels (GIRKs), voltage-gated Ca^{2+} channels (VGCCs) and adenylyl cyclases (ACs). The K^+ channel tetramerization domain-containing (KCTD) proteins were shown to be auxiliary subunits of $GABA_B$ receptors that constitutively bind to the receptor via $GABA_{B2}$ and regulate receptor signaling in a KCTD-subtype specific manner (Schwenk et al., 2010). The aim of this thesis was to better understand the KCTD12-mediated modulation of $GABA_B$ receptor signaling as well as to explore new mechanisms of receptor fine-tuning.

The first part of this thesis uncovers the mechanism of $GABA_B$ receptor-activated K^+ current desensitization induced by KCTD12. Besides the constitutive binding of all KCTDs to the G-protein, selectively KCTD12 has a second binding site on the activated Gβγ subunit. A switch in binding uncouples Gβγ from the K^+ channels and induces K^+ current desensitization. Native KCTD12 associates exclusively with $GABA_B$ receptors rendering this mechanism receptor specific. (Turecek et al., 2014)

In the second part, we investigated the effects of $GABA_B$ receptor phosphorylation on KCTD12-induced K^+ current desensitization. Phosphorylation of serine-892 (S892) in $GABA_{B2}$ by protein kinase A (PKA) was previously described as a rather slow mechanism to regulate $GABA_B$ receptor desensitization (Couve et al., 2002). In contrast, KCTD12-induced desensitization of $GABA_B$ receptor-activated K^+ currents represents a fast form of desensitization. We show that both mechanisms of desensitization influence each other. S892 phosphorylation slows KCTD12-induced K^+ current desensitization by rearranging KCTD12 at the receptor. In turn, KCTD12 promotes tonic S892 phosphorylation by binding to $GABA_B$ receptors. This cross-regulation renders the signaling of $GABA_B$ receptors more precise and highly dependent on both the expression of KCTD12 and the activity of PKA. (Adelfinger et al., 2014)

The third part reveals that all KCTDs exert marginal allosteric influence on the ligand binding affinity of $GABA_B$ receptors. However, we show that KCTD8, in contrast to KCTD12 and KCTD16, reduces the basal G-protein activation of $GABA_B$ receptors. (Rajalu et al., 2014)

The fourth part summarizes an ongoing project in which we investigate the effects of 14-3-3 proteins and cullin3 (CUL3) on $GABA_B$ receptor signaling. 14-3-3 ε and γ specifically interact with KCTD16 through its H2-domain. This binding regulates the expression of KCTD16 and $GABA_{B2}$ resulting in altered Gα-signaling of $GABA_B$ receptors. Furthermore, we report a specific interaction between KCTD16 and CUL3, which is controlled by 14-3-3 proteins. In summary, we propose that 14-3-3 proteins determine the rate of lysosomal $GABA_B$ receptor degradation, which is induced upon binding of KCTD16 and CUL3. (Adelfinger et al., in preparation)

Finally, in collaboration with the group of Jan Siemens from Heidelberg we studied how $GABA_B$ receptors reciprocally counteract the sensitization of the capsaicin receptor TRPV1. (Hanack et al., in preparation)

II. ABBREVIATIONS

AC	adenylyl cyclase
AMP	adenosine monophosphate
AMPK	AMP-dependent protein kinase
AP	action potential
BRET	bioluminescence resonance energy transfer
BTB	Bric-a-brac, Tramtrack, Broad-complex
cAMP	cyclic adenosine monophosphate
CA1	Cornu Ammonis area 1
CaR	calcium-sensing receptor
$Ca_V2.1/2.2$	voltage-gated P/Q- and N-type Ca^{2+} channels
CNS	central nervous system
COPI	coat protein complex I
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ESCRT	endosomal sorting complex required for transport
GABA	gamma-amino butyric acid
$GABA_A$	gamma-amino butyric acid receptor type A
$GABA_B$	gamma-amino butyric acid receptor type B
$GABA_{B2}^{-/-}$	$GABA_{B2}$ deficient
$GABA_C$	gamma-amino butyric acid receptor type C
GAD	glutamate decarboxylase
GDP	guanosine diphosphate
GIRK	G-protein coupled inwardly rectifying K^+ channels
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
H1, H2	homology domain 1 and 2
IPSC	inhibitory postsynaptic current
KCTD	K^+ channel tetramerization domain containing

NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA-type glutamate receptors
PAM	positive allosteric modulator
PKA	cyclic AMP-dependent protein kinase A
POZ	Pox virus and Zinc finger
T1	tetramerization domain
VFTM	venus flytrap module
VGCC	voltage-gated Ca^{2+} channels
WT	wild-type

III. PREFACE

This thesis is based on the following manuscripts that are published or in preparation. Asterisk (*) indicate equal contributions by the authors.

Auxiliary $GABA_B$ receptor subunits uncouple G protein βγ subunits from effector channels to induce desensitization

Rostislav Turecek, Jochen Schwenk, Thorsten Fritzius, Klara Ivankova, Gerd Zolles, Lisa Adelfinger, Valerie Jacquier, Valerie Besseyrias, Martin Gassmann, Uwe Schulte, Bernd Fakler and Bernhard Bettler

Neuron 2014 Jun 4;82(5):1032-44

$GABA_B$ receptor phosphorylation regulates KCTD12-induced K^+ current desensitization

Lisa Adelfinger*, Rostislav Turecek*, Klara Ivankova, Anders A. Jensen, Stephen J. Moss, Martin Gassmann and Bernhard Bettler

Biochemical Pharmacology 2014 Oct 1;91(3):369-79

Pharmacological characterization of $GABA_B$ receptor subtypes assembled with auxiliary KCTD subunits

Mathieu Rajalu, Thorsten Fritzius, Lisa Adelfinger, Valerie Jacquier, Valerie Besseyrias, Martin Gassmann and Bernhard Bettler

Neuropharmacology, 2014 Sep 6. pii: S0028-3908(14)00303-7. doi: 10.1016/j.neuropharm.2014.08.020

$GABA_B$ receptor signaling is controlled by complex formation of KCTD16, cullin3 and 14-3-3 proteins

Lisa Adelfinger, Valerie Besseyrias, Martin Gassmann and Bernhard Bettler

in preparation

1 INTRODUCTION

1.1 GABA and GABA receptors

In 1950, Roberts and Frankel described γ-aminobutyric acid (GABA) for the first time as a highly abundant amino acid in the brain (Roberts and Frankel, 1950). Later GABA was shown to be the major inhibitory neurotransmitter in the central nervous system, besides L-glutamate being the major excitatory neurotransmitter in the brain (Curtis et al., 1959). As both neurotransmitters are involved in a wide range of neurological processes and functions, an imbalance in excitation and inhibition is often the cause for neurological and psychiatric disorders, including epilepsy, bipolar disorders, depression or anxiety.

GABA is synthesized from its precursor L-glutamate by glutamate decarboxylase (Roberts and Frankel, 1950). GABAergic nerve terminals release GABA into the synaptic cleft, where it binds to GABA receptors localized at pre- or postsynaptic membranes. Its action is mediated by two main classes of receptors, the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. $GABA_C$ receptors were described as a third class of GABA receptors mainly expressed in the retina (Chebib, 2004). However, nowadays they are rather considered to be a subtype of $GABA_A$ receptors as they share high similarity in structure and function with $GABA_A$ receptors.

Ionotropic $GABA_A$ receptors belong to the superfamily of ligand-gated ion channels that comprise glycine receptors, nicotinic acetylcholine receptors and 5-HT_3 serotonin receptors. They are pentameric transmembrane receptors that are formed from a repertoire of 16 subunits (α1-6, β 1-3, γ1-3, δ, ε, π, and θ) (Mohler, 2006). The subunit assembly determines the physiology and localization of $GABA_A$ receptors. Activation of $GABA_A$ receptors results in neuronal inhibition as they increase the membrane conductance of the postsynapse thereby decreasing the probability of action potential formation (Olsen and DeLorey, 1999). $GABA_A$ receptor ligands are commonly used as therapeutics for psychiatric and neurological disorders, like anxiety or epilepsy (Foster and Kemp, 2006). Barbiturates and benzodiazepines are two examples of widely used drugs that act anxiolytic, anticonvulsant and sedative by enhancing GABAergic transmission through $GABA_A$ receptors (Olsen and DeLorey, 1999). Whereas $GABA_A$ receptors provoke fast inhibitory postsynaptic currents (IPSCs), a slow form of IPSCs is mediated by metabotropic $GABA_B$ receptors.

1.2 The $GABA_B$ receptors

Structure and Function

Metabotropic $GABA_B$ receptors belong to the class C of G protein-coupled receptors (GPCRs), which include calcium-sensing receptors, metabotropic glutamate receptors, taste receptors type 1 and some orphan receptors (Foord et al., 2005). Functional $GABA_B$ receptors are obligate heteromers that assemble from two principal subunits, $GABA_{B1}$ and $GABA_{B2}$ (Jones et al., 1998, Kaupmann et al., 1998, White et al., 1998, Kuner et al., 1999, Ng et al., 1999). Receptor heterogeneity is based on two splice variants of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$, which differ in their N-terminus by a pair of sushi domains unique to $GABA_{B1a}$ (Blein et al., 2004). Both $GABA_{B1}$ and $GABA_{B2}$ subunits contain a long

intracellular tail, a seven transmembrane domain and a large extracellular venus fly-trap domain (VFTD) that harbors the ligand-binding site (Galvez et al., 1999, Galvez et al., 2000). Despite a high degree of homology, both subunits exert unique functions and characteristics. $GABA_{B1}$ provides the binding site for GABA, $GABA_{B2}$ increases the agonist affinity on $GABA_{B1}$ and mediates receptor signaling by coupling to the G-protein (Malitschek et al., 1999, Galvez et al., 2000, Liu et al., 2004). Unique to $GABA_{B1}$ is the RSRR retention signal for the endoplasmic reticulum (ER), which is masked by the interaction with $GABA_{B2}$ via their intracellular coiled-coil domain (Margeta-Mitrovic et al., 2000, Pagano et al., 2001, Gassmann et al., 2005). Accordingly, only correctly formed $GABA_{B1/2}$ heterodimers translocate to the cell surface and represent functional receptors. Ligand binding to $GABA_{B1}$ causes a conformational rearrangement of the VFTD dimer, which is required for receptor activation and G-protein coupling by $GABA_{B2}$ (Rondard et al., 2008, Rondard et al., 2011). As heteromerization is obligate, ablation of either $GABA_{B1}$ or $GABA_{B2}$ eliminates any physiological responses of $GABA_B$ receptors in mice (Schuler et al., 2001, Gassmann et al., 2004).

Heterotrimeric G-proteins are the molecular switches in signal transduction in response to the activation of GPCRs (Oldham and Hamm, 2008). They are composed of three subunits, α, β and γ. The α-subunit cycles between an inactive GDP-bound state and an active GTP-bound state. The heterotrimeric, inactive G-protein is bound to the receptor and dissociates into the GTP-bound α-subunit and the βγ-dimer upon receptor activation. Consequently, both subunits are able to interact with their downstream effectors. The signal is terminated on hydrolysis of GTP to GDP by the GTPase activity of the Gα, which is promoted by regulators of G-protein signaling (RGS) proteins.

Four main classes of heterotrimeric G-proteins are described based on the diversity of the Gα subunit: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12}$ (Simon et al., 1991). $GABA_B$ receptors are coupled to $G\alpha_{i/o}$-type G-proteins (Campbell et al., 1993, Greif et al., 2000). Free $G\alpha_{i/o}$ inhibits adenylyl cyclase, which accordingly decreases intracellular cAMP levels and the activity of PKA. In presynaptic compartments, low levels of cAMP prevent spontaneous neurotransmitter release by restricting vesicle fusion (Sakaba and Neher, 2003, Rost et al., 2011). Postsynaptically, reduced PKA activity inhibits the Ca^{2+} permeability of NMDA-type glutamate receptors (NMDARs), disinhibits TREK2 channels and affects gene expression (Fukui et al., 2008, Deng et al., 2009, Chalifoux and Carter, 2010, Schwirtlich et al., 2010). The βγ-subunits of the G-protein, on the other hand, limit vesicle fusion and evoked Ca^{2+}-dependent neurotransmitter release at the presynapse by inhibiting voltage-gated P/Q- ($Ca_V2.1$) and N-type ($Ca_V2.2$) Ca^{2+} channels (Couve et al., 2000, Bowery et al., 2002, Bettler et al., 2004, Yoon et al., 2007, Wells et al., 2012). Postsynaptically released Gβγ hyperpolarizes the cell by opening G protein-activated inwardly rectifying potassium (GIRK) channels, which induces slow inhibitory postsynaptic currents (slow IPSCs) and inhibits neuronal excitability (Couve et al., 2000, Bowery et al., 2002, Bettler et al., 2004, Leung and Peloquin, 2006).

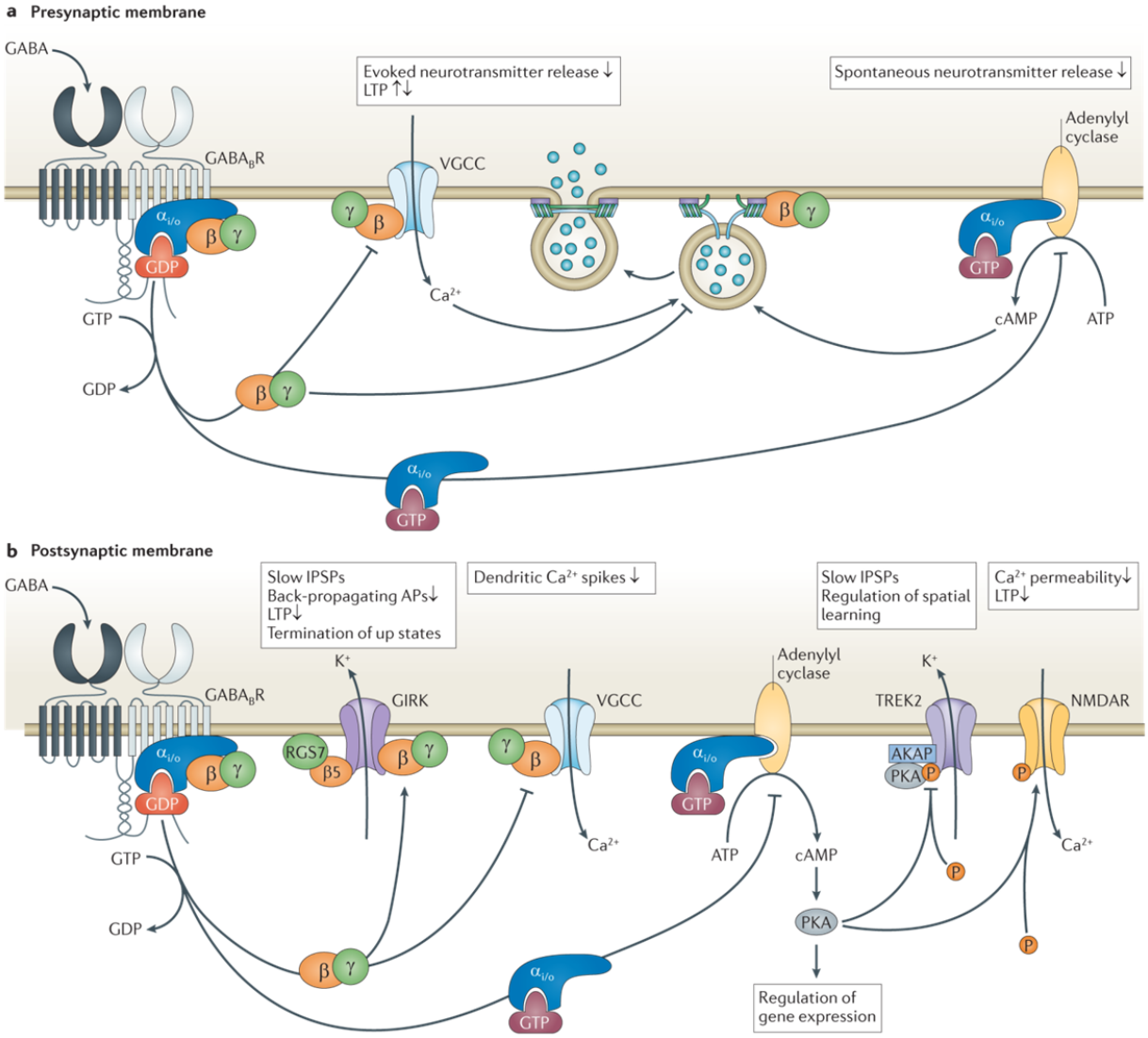

Figure 1: **Pre- and postsynaptic signaling pathways of GABA$_B$ receptors. a¦** Presynaptic GABA$_B$ receptors activate Gα$_{i/o}$-type G-proteins. The α-subunit inhibits the activity of adenylyl cyclase, which decreases intracellular cyclic adenosine monophosphate (cAMP) levels. Low cAMP prevents spontaneous neurotransmitter release (Sakaba and Neher, 2003, Rost et al., 2011). Released βγ-subunits of the G-protein negatively couple to voltage-gated Ca^{2+} channels (VGCC) resulting in low intracellular Ca^{2+} levels, which inhibits evoked neurotransmitter release. This, in turn, regulates long-term potentiation (LTP) processes (Davies et al., 1991, Shaban et al., 2006, Vigot et al., 2006). **b¦** Activation of postsynaptic GABA$_B$ receptors inhibits adenylyl cyclase and protein kinase A (PKA), which affects gene regulation, alleviates tonic inhibition of TREK2 channels and activates NMDA-type glutamate receptors (NMDARs) (Fukui et al., 2008, Deng et al., 2009, Chalifoux and Carter, 2010, Schwirtlich et al., 2010). Released βγ opens G protein-coupled inwardly rectifying potassium (GIRK) channels resulting in slow inhibitory postsynaptic potentials (IPSPs), less back-propagating action potentials (APs) and the termination of up states (Scanziani, 2000, Koch and Magnusson, 2009, Mann et al., 2009, Isaacson and Scanziani, 2011). They further inhibit VGCCs which prevents dendritic Ca^{2+} spikes (Perez-Garci et al., 2006, Chalifoux and Carter, 2011). Figure from (Gassmann and Bettler, 2012).

Distribution and Localization

$GABA_B$ receptors are widely distributed in the central nervous system. They are abundantly expressed in most neuronal cell populations and to a lower extend in non-neuronal cells (Charles et al., 2003). Brain areas of high $GABA_B$ receptor expression are the cerebellum, cortex, thalamus and hippocampus (Durkin et al., 1999, Fritschy et al., 1999, Margeta-Mitrovic et al., 1999). Electron microscopy and $GABA_B$-specific antibodies enabled localization studies in defined subcellular compartments (Kaupmann et al., 1998, Kulik et al., 2002, Lopez-Bendito et al., 2002, Kulik et al., 2003). $GABA_B$ receptors are localized on presynaptic and, more abundantly, on postsynaptic membranes. Presynaptic $GABA_B$ receptors were mostly found on extrasynaptic membranes and occasionally over presynaptic membrane specializations of glutamatergic and, to a lesser extent, of GABAergic terminals. Their presynaptic expression plays a crucial role in regulating the neurotransmitter release from glutamatergic synapses (heteroreceptors) and GABAergic synapses (autoreceptors)(Kulik et al., 2002). Postsynaptic $GABA_B$ receptors are enriched extrasynaptically on spines around putative glutamatergic synapses and evenly distributed on dendritic shafts of principal cells contacted by GABAergic boutons. The apparent restriction of $GABA_B$ receptors to extrasynaptic compartments accounts for receptor activation by GABA spillover from simultaneously active GABAergic cells (Scanziani, 2000).

The distribution of $GABA_B$ receptors is furthermore regulated by the heterogeneity of its principal subunits. Whereas $GABA_{B(1a,2)}$ receptors are mainly localized to presynaptic compartments, $GABA_{B(1b,2)}$ receptors are found more prominent in postsynaptic membranes (Vigot et al., 2006). This subunit-specific division of $GABA_B$ receptor expression indicates a higher contribution of $GABA_{B(1a,2)}$ receptors to heteroreceptor function (Guetg et al., 2009). In contrast, both $GABA_{B1}$ subunits contribute equally to autoreceptor function on GABAergic terminals. A summary of phenotypes of $GABA_B$ receptor principal subunit mutant mice can be found in (Gassmann and Bettler, 2012).

KCTD proteins, the auxiliary subunits of $GABA_B$ receptors

The studies of recombinant $GABA_B$ receptors displayed different receptor characteristics compared to native $GABA_B$ receptors. Affinity-purification of native $GABA_B$ receptor complexes followed by mass spectrometry analysis revealed a number of interacting proteins that may modify $GABA_B$ receptor responses and may account for the discrepancies between native and recombinant receptor responses (Bartoi et al., 2010, Schwenk et al., 2010). These studies identified members of the K^+ channel tetramerization domain-containing (KCTD) protein family, namely KCTD8, 12, 12b and 16, to be associated with native $GABA_B$ receptors. These cytosolic proteins bind $GABA_B$ receptors with their conserved N-terminal tetramerization (T1) domain, which is highly homologous to the T1 domain of voltage-gated K^+ channels (Schwenk et al., 2010). It contains a BTB (Bric-a-brac, Tramtrack, Broad-complex) domain, also termed POZ (Pox virus and Zinc finger) domain, that is required for self-association and binding to other proteins (Godt et al., 1993, Bardwell and Treisman, 1994, Zollman et al., 1994). Some BTB-containing proteins were found to be important for ion channel function, cytoskeletal or transcriptional regulation and protein degradation (Melnick et al., 2000, Furukawa et al., 2003, Stogios et al., 2005). All four KCTD proteins have a sequence-related H1 homology domain but only KCTD8 and KCTD16 have an additional sequence-related H2 homology domain (Schwenk et al., 2010). These

domains do not share obvious sequence similarities with other proteins that would indicate a certain function.

All KCTDs were shown to shorten the rise time of $GABA_B$ receptor-activated K^+ currents (Schwenk et al., 2010). The most prominent effect on $GABA_B$ receptor signaling is induced by KCTD12 and KCTD12b. Both subunits were shown to strongly desensitize $GABA_B$ receptor-activated K^+ currents and $GABA_B$ receptor-mediated inhibition of voltage-gated Ca^{2+} channel (VGCC) currents (Schwenk et al., 2010). Accordingly, KCTD12 knock-down in mice exhibits significantly less desensitization of $GABA_B$ receptor-activated K^+ currents confirming the recombinant effects of KCTD12 in vivo (Schwenk et al., 2010). It is interesting to note that KCTD12 promotes desensitization while at the same time up-regulates the cell surface expression of $GABA_B$ receptors resulting in a higher baclofen-induced K^+ current density (Ivankova et al., 2013). As a consequence of both effects, it is suggested that KCTD12 increases the temporal precision of $GABA_B$ receptor signaling.

Trafficking, desensitization and degradation

ER export

Cell surface expression of $GABA_B$ receptors is controlled by the export of $GABA_{B1}$ from the ER. While $GABA_{B2}$ is able to independently translocate to the membrane, $GABA_{B1}$ is retained in the ER via its ER retention signal. The coat protein complex I (COPI) recognizes such signals thereby mediating the retrieval of $GABA_{B1}$ from the *cis*-Golgi back to the ER (Brock et al., 2005). Some cases have been reported, where 14-3-3 proteins interfere with the ER retrieval by competing with this interaction. However, forward trafficking of $GABA_{B1}$ was shown to take place independent of 14-3-3 proteins (Brock et al., 2005). It is, however, dependent on the heterodimerization with $GABA_{B2}$, which masks the ER retention signal and promotes ER export (Margeta-Mitrovic et al., 2000, Pagano et al., 2001, Gassmann et al., 2005). The ER expression of $GABA_{B2}$ is hence the limiting factor for receptor trafficking to the cell surface.

Desensitization

Once the receptors are localized at the cell membrane, prolonged receptor activation initiates the termination of receptor responses to protect the cell from overstimulation. This phenomenon is referred to as desensitization (Sodickson and Bean, 1996, Wetherington and Lambert, 2002, Sickmann and Alzheimer, 2003, Cruz et al., 2004). For most GPCRs, this event includes phosphorylation by G protein-coupled receptor kinases (GRKs) followed by an arrestin-mediated internalization (Ferguson, 2001, Gainetdinov et al., 2004). Internalized receptors are subsequently de-phosphorylated to be recycled or finally degraded in lysosomes. The classical mechanism of GPCR desensitization, however, does not apply for $GABA_B$ receptors. Although GRKs induce desensitization of $GABA_B$ receptors, this was shown to be independent of their kinase activity. GRK4 induces a phosphorylation-independent $GABA_B$ receptor desensitization by directly interacting with the receptor (Perroy et al., 2003, Kanaide et al., 2007, Ando et al., 2011). Also GRK2 induces desensitization independent of its kinase activity by scavenging Gβγ from

the GIRK channels (Raveh et al., 2010, Turecek et al., 2014). Besides these phosphorylation-independent mechanisms, $GABA_B$ receptor desensitization can be further induced by phosphorylation-dependent mechanisms. An example is the interaction of NEM-sensitive fusion (NSF) protein with the $GABA_B$ receptor, which promotes protein kinase C (PKC)-mediated receptor phosphorylation and subsequent desensitization (Pontier et al., 2006). On the contrary, receptor phosphorylation can even counteract its desensitization. The phosphorylation of $GABA_{B2}$ S783 or S892 by AMP-dependent protein kinase (AMPK) and PKA, respectively, enhance $GABA_B$ receptor-activated GIRK currents by increasing the number of membrane-bound receptors (Couve et al., 2002, Kuramoto et al., 2007). Together, these studies underlay the importance of phosphorylation events on the regulation of $GABA_B$ receptor desensitization.

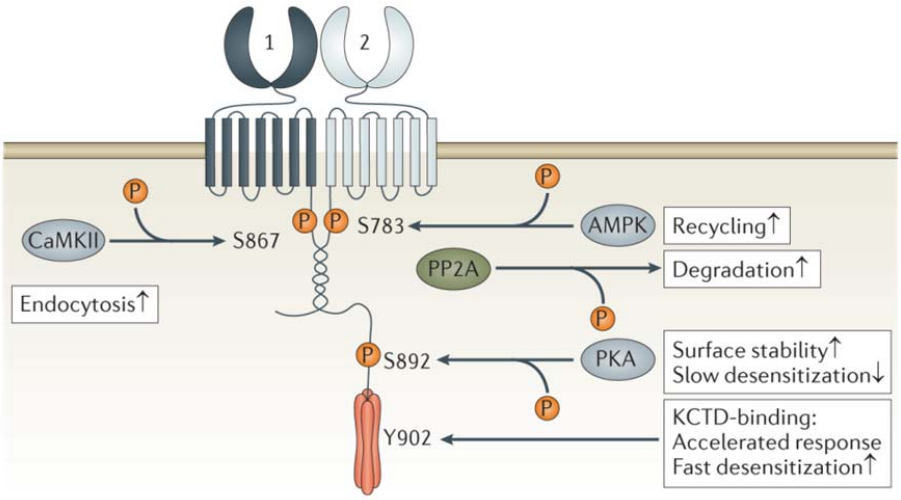

Figure 2: **Phosphorylation of serine residues on principal $GABA_B$ receptor subunits modulates surface expression and effector coupling.** Phosphorylation of S867 in $GABA_{B1}$ by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) increases $GABA_B$ receptor endocytosis and inhibits $GABA_B$ receptor-mediated activation of G protein-activated inwardly rectifying potassium channels (GIRKs) (Guetg et al., 2010). AMP-dependent protein kinase (AMPK)-mediated phosphorylation and protein phosphatase 2A (PP2A)-mediated de-phosphorylation of S783 in $GABA_{B2}$ promotes receptor recycling and degradation, respectively (Maier et al., 2010, Terunuma et al., 2010). Phosphorylation of S892 in $GABA_{B2}$ by protein kinase A (PKA) increases surface stability and thereby $GABA_B$ receptor-mediated activation of GIRK channels (Couve et al., 2002). Figure from (Gassmann and Bettler, 2012).

Endocytosis

The endocytic pathway is a process that precisely controls the activity of GPCRs. It determines whether GPCRs are sorted to endosomes for recycling or to lysosomes for degradation. GPCRs are internalized from the cell membrane by constitutive or agonist-induced endocytosis, the latter allowing for fast signal termination. It is still a controversy whether $GABA_B$ receptors undergo agonist-induced internalization. Whereas some studies describe the endocytosis of $GABA_B$ receptors upon sustained agonist treatment (Gonzalez-Maeso et al., 2003, Laffray et al., 2007), a number of other publication could not confirm these results (Fairfax et al., 2004, Grampp et al., 2007, Grampp et al., 2008, Vargas et al., 2008). Constitutive

endocytosis, on the other hand, is a well-accepted mechanism for $GABA_B$ receptors. Both heterologous and native $GABA_B$ receptors constitutively internalize via the clathrin- and dynamin-dependent pathway that is controlled by glutamate (Grampp et al., 2007, Grampp et al., 2008, Vargas et al., 2008, Wilkins et al., 2008, Pooler et al., 2009). $GABA_B$ receptors were shown to internalize as dimers and their rate of internalization is regulated by the $GABA_{B2}$ subunit (Hannan et al., 2011). Following internalization, $GABA_B$ receptors are sorted either to endosomes for recycling or to lysosomes for degradation. Receptor recycling was found to be accelerated by the $GABA_B$ receptor agonist baclofen (Laffray et al., 2007, Grampp et al., 2008). Constitutive internalization and rapid recycling generate a high level of intracellular receptors that can be instantly inserted into the cell membrane. Increasing the rate of recycling without changing the rate of internalization (Benke et al., 2012) helps to keep the energy expenditure of a cell low as both, internalization and recycling, are energy-intensive processes.

Degradation

After endocytosis, GPCRs are either re-used or degraded. For recycling, receptors are sorted to endosomes and subsequently re-inserted into the membrane. For degradation, receptors are directed to lysosomes, the major compartment of a cell for the degradation of membrane receptors. Lysosomal degradation is initiated by fusion of endocytic vesicles containing the receptors with early endosomes followed by their maturation to late endosomes. The endosomal sorting complex required for transport (ESCRT) machinery binds K63-linked ubiquitinated proteins and targets them to endosomes (Raiborg and Stenmark, 2009). Finally, late endosomes fuse with lysosomes, where the ingested receptors are degraded (Futter et al., 1996, Bright et al., 1997, Mullock et al., 1998, Ward et al., 2000). $GABA_B$ receptors were shown to be present in early and late endosomes and to be degraded in lysosomes dependent on the ESCRT machinery (Grampp et al., 2007, Grampp et al., 2008, Kantamneni et al., 2008, Hannan et al., 2011).

$GABA_B$ receptors are abundant on glutamatergic synapses and precisely control glutamate release (Kulik et al., 2006, Guetg et al., 2009). It is therefore not surprising that glutamate in turn regulates the expression of $GABA_B$ receptors by controlling their postendocytic sorting (Vargas et al., 2008, Maier et al., 2010). Application of glutamate to cortical neurons decreases the amount of cell surface $GABA_B$ receptors by shifting the balance from recycling to degradation (Maier et al., 2010). Furthermore, a reciprocal signaling crosstalk between $GABA_B$ receptors and NMDARs was observed. While $GABA_B$ receptors inhibit the Ca^{2+} permeability of NMDARs, NMDARs mediate $GABA_B$ receptor internalization by phosphorylation of S867 in $GABA_{B1}$ and S783 in $GABA_{B2}$ (Morrisett et al., 1991, Otmakhova and Lisman, 2004, Chalifoux and Carter, 2010, Guetg et al., 2010, Terunuma et al., 2010). This crosstalk determines the receptor contribution to postsynaptic signaling which may be relevant for diseases that depend on both $GABA_B$ receptors and NMDARs, like obsessive compulsive disorders (Richter et al., 2011).

For several years, lysosomal degradation was the only known degradation pathway that regulates the expression of $GABA_B$ receptors. Very recently, Zemoura et al. discovered that cell surface expression of $GABA_B$ receptors is also controlled by the proteasomal degradation pathway (Zemoura et al., 2013, Zemoura and Benke, 2014). Proteasomal degradation requires the covalent attachment of ubiquitin to the substrate, which is bound to a E3 ubiquitin-ligase (for a review see (Glickman and Ciechanover,

2002)). Ubiquitin is activated by the ubiquitin-activation enzyme E1 and transferred from E1 to the substrate by the ubiquitin-conjugating enzyme E2. The proteasome recognizes explicitly K48-linked polyubiquitinated proteins (Chau et al., 1989, Finley et al., 1994), whereas K63-linked polyubiquitin chains serve as non-proteolytic signal (Deng et al., 2000). The proteasomal degradation pathway serves as quality control of ER membrane proteins or proteins that cross the ER membrane. Abnormal or misfolded receptors are removed from the ER through the ER-associated degradation (ERAD) (Vembar and Brodsky, 2008). $GABA_B$ receptors are K48-linked polyubiquitinated on the C-terminus of the $GABA_{B2}$ subunit and interact with components of the ERAD machinery, which controls the amount of $GABA_B$ receptors (Zemoura et al., 2013). Finally, proteasomal degradation of $GABA_B$ receptors is controlled by neuronal activity (Zemoura and Benke, 2014), which may have a major influence on homeostatic synaptic plasticity.

1.3 The 14-3-3 proteins

Structure, expression and binding sites

14-3-3 proteins were first described in 1967 as acidic dimeric proteins and their name derived from their elution and migration profile on DEAE-cellulose chromatography and starch gel electrophoresis (Moore and Perez, 1967, Ichimura et al., 1988, Martin et al., 1993). They are a highly conserved protein family and abundant in all eukaryotic organisms. Multiple isoforms are found in many organisms, with seven isoforms in mammals (β, γ, ε, ζ, η, τ, σ) two of them (β and γ) having a phosphorylated form (α and δ) (Aitken et al., 1995). They are expressed in almost all tissues, including brain, heart, liver and testes (Boston et al., 1982, Celis et al., 1990). 14-3-3 proteins are mainly localized in the cytoplasm but also found at the plasma membrane, ER, Golgi and nucleus (Celis et al., 1990, Leffers et al., 1993, Freed et al., 1994, Martin et al., 1994, Fanger et al., 1998, Tang et al., 1998, Garcia-Guzman et al., 1999). All 14-3-3 proteins, except 14-3-3 σ, form homo- and heterodimers, which allows the binding of two substrates at a time (Benzinger et al., 2005, Wilker et al., 2005, Gardino et al., 2006). 14-3-3 proteins interact with more than 200 proteins involved in almost every cellular process, like signal transduction, apoptosis, protein trafficking, protein degradation or endocytosis. Although 14-3-3 proteins mostly recognize and bind phosphorylated peptides of their binding partners, some studies report phosphorylation-independent binding of 14-3-3 to their substrates (Yaffe et al., 1997). Three main consensus sequences are described as 14-3-3 binding motifs. The mode I binding site is R-S-X-pS/pT-X-P, the mode II binding site is R-X-F/Y-X-pS/pT-X-P, where pS/pT represents a phosphoserine or phosphothreonine and X is any residue (Muslin et al., 1996, Yaffe et al., 1997, Rittinger et al., 1999). The third binding motif was found years later to be located at the very C-terminus of some proteins containing an R-X-X-pS/pT-X-COOH sequence (Coblitz et al., 2005, Shikano et al., 2005). Still, it is important to note that some 14-3-3 interactions do not involve any of the above mentioned motifs (Petosa et al., 1998, Masters et al., 1999, Wang et al., 1999, Zhai et al., 2001, Henriksson et al., 2002).

Function

14-3-3 proteins play an important role in a variety of cellular processes so their functions are described to be manifold. A predominant function of 14-3-3 proteins is the translocation of proteins from the nucleus to the cytoplasm or from the ER to the cell membrane. 14-3-3-mediated nuclear export to the cytoplasm was shown for class II histone deacetylases (HDACs), the cyclin-dependent kinase inhibitor p27 and the human telomerase reverse transcriptase (hTERT) (Seimiya et al., 2000, McKinsey et al., 2001, Sekimoto et al., 2004). The ER export of membrane proteins is probably the best described function of 14-3-3 proteins (Nufer and Hauri, 2003, Coblitz et al., 2005). Membrane proteins contain an ER retention signal - internal RXR or C-terminal KKXX - that prevents cell surface expression of unassembled or misfolded proteins. The binding of COPI to either of these signals ensures the retrieval of cargo from the *cis*-Golgi to the ER (Bonifacino and Lippincott-Schwartz, 2003, Yuan et al., 2003, Lee et al., 2004). 14-3-3 proteins compete with this interaction, which promotes the forward trafficking of their binding partners (O'Kelly et al., 2002, Rajan et al., 2002, Yuan et al., 2003, Zuzarte et al., 2009) and eventually up-regulates cell surface expression (Godde et al., 2006, Heusser et al., 2006, Okamoto and Shikano, 2011, Cho et al., 2014). However, cell surface expression can be dependent on COPI but independent on 14-3-3 proteins as shown for the $GABA_{B1}$ subunit (Brock et al., 2005). Another function of 14-3-3 proteins is the regulation of protein activity and stability. Binding of 14-3-3 can inhibit the activity of proteins, as shown for the big mitogen-activated protein kinase 1 (BMK1), or enhance it, as shown for the plasma membrane H^+-ATPase, protein kinase C, human tryptophan hydroxylase 2, T-lymphoma invasion and metastasis protein (Tiam1), serotonin N-acetyltranserase (AANAT) or $SCF^{Fbx}4$ (Van Der Hoeven et al., 2000, Obsil et al., 2001, Kanczewska et al., 2005, Winge et al., 2008, Woodcock et al., 2009, Barbash et al., 2011).

The interaction of 14-3-3 proteins with a number of GPCRs provides them with a pivotal role in the regulation of signal transduction. β_1-adrenergic receptors (β_1ARs) deficient in 14-3-3 ε-binding were found to activate Kv11.1 K^+ channels in contrast to wild-type receptors that inhibit these channels (Tutor et al., 2006). It is suggested that Kv11.1 - also capable of 14-3-3 ε binding - and β_1AR compete for the same pool of 14-3-3 ε, which regulates the β_1AR/Kv11.1 signaling. Another member of the 14-3-3 family, 14-3-3 τ, binds the human follitropin receptor (FSHR) resulting in decreased follitropin-induced cAMP accumulation (Cohen et al., 2004). 14-3-3 θ and ζ bind the calcium-sensing receptor (CaR) and attenuate its Rho kinase signaling likely through masking the CaR binding site for Rho GTPase (Arulpragasam et al., 2012). The loss of 14-3-3 ζ binding to the human thromboxane receptor (TP), on the other hand, was shown to decrease TR agonist-induced activation of ERK as 14-3-3 ζ is thought to function as scaffold protein between TP and Raf-1 to ensure signal transduction (Yan et al., 2013). Finally, 14-3-3 ζ was shown to impair $GABA_B$ receptor signaling (Laffray et al., 2012). The binding of 14-3-3 ζ to $GABA_{B1}$ dissociates the $GABA_{B1/2}$ dimer accompanied with a strong reduction in baclofen-induced activation of K^+ channels. Taken together, despite their ubiquitous expression and their various interaction partners, 14-3-3 proteins show very drastic but specific effects on many important cellular processes dependent on their isoform expression and subcellular localization.

1.4 Aim of the thesis

$GABA_B$ receptors are crucial components of signaling pathways in the brain. Their correct function and a precise temporal and spatial regulation are therefore indispensable for proper brain function. Transgenic mice lacking either of the $GABA_B$ receptor subunits display severe neurological phenotypes ranging from spontaneous seizures, hyperalgesia, hyperlocomotor activity and memory impairment to anxiogenic and antidepressant behavior (Schuler et al., 2001, Gassmann et al., 2004, Mombereau et al., 2004a, Mombereau et al., 2004b, Mombereau et al., 2005, Vacher et al., 2006). Similarly, overexpression of $GABA_B$ receptor subunits induces phenotypes like atypical absence epilepsy (Wu et al., 2007, Stewart et al., 2009). Together, these studies emphasize that perturbations of $GABA_B$ receptor signaling have far-reaching consequences and that approaches to fine-tune $GABA_B$ receptor responses need to be explored as they have an enormous therapeutic potential for a variety of neurological disorders. The aim of this thesis was therefore to study different types of $GABA_B$ receptor regulation.

The first part of this study aims at understanding the molecular mechanism of KCTD12-induced desensitization of $GABA_B$ receptor-activated K^+ currents by using proteomic and electrophysiological approaches (2.1). To achieve this goal, it was important to determine at which level the desensitization operates (receptor, G-protein or channels) and to compare it with similar forms of fast desensitization. We further addressed the question whether native KCTD12-induced desensitization is specific for $GABA_B$ receptors or whether KCTD12 desensitizes K^+ currents activated by other GPCRs.

In a second project we study the interplay between the slow, phosphorylation-induced and the fast, KCTD12-induced desensitization of $GABA_B$ receptors. Slow desensitization is mediated by receptor activation and subsequent inhibition of protein kinase A (PKA). As PKA phosphorylation of $GABA_{B2}$ serine-892 (S892) enhances receptor stability at the plasma membrane, reduced PKA activity promotes receptor internalization and desensitization. The proximity of S892 and the KCTD12 binding-site Y902 on $GABA_{B2}$, led us to the hypothesis that $GABA_{B2}$ phosphorylation might influence KCTD12-induced desensitization (2.2). Therefore, we tested S892-phosphorylation mutants of $GABA_{B2}$ in their properties to activate K^+ currents and to bind KCTD12. We further used genetic mouse models to confirm our data in vivo. Finally, we investigated whether this regulation might also exist in the opposite direction and tested the phosphorylation of $GABA_{B2}$ in the presence and absence of KCTD12.

Furthermore, knowledge and skills that have been acquired in these projects are used to study the allosteric effects of KCTDs on pharmacological properties of $GABA_B$ receptors (2.3). This project revealed for the first time an implication of KCTD8 in $GABA_B$ receptor signaling.

Lastly, an ongoing project assesses a possible relevance of KCTD16 for the signaling of $GABA_B$ receptors (2.4). To achieve this goal we characterized the interaction between KCTD16, 14-3-3 proteins and cullin3 (CUL3). These binding studies revealed a functional effect of 14-3-3 proteins on the expression of KCTD16 as well as of $GABA_B$ receptors. They further seem to interfere with the binding of CUL3 to KCTD16, another newly discovered interaction. CUL3 is part of the cullin-RING ubiquitin ligase complex that mediates protein ubiquitination and degradation. Our recent data indicate that CUL3 is part of the $GABA_B$ receptor complex and might therefore promote the lysosomal degradation of membrane $GABA_B$ receptors, a process that is not yet fully understood.

2 PUBLICATIONS

2.1 Auxiliary $GABA_B$ receptor subunits uncouple G protein βγ subunits from effector channels to induce desensitization

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Personal contribution

BRET measurements

Data analysis

Design of figures during revision

CellPress

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Article

Auxiliary $GABA_B$ Receptor Subunits Uncouple G Protein βγ Subunits from Effector Channels to Induce Desensitization

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SUMMARY

Activation of K^+ channels by the G protein βγ subunits is an important signaling mechanism of G-protein-coupled receptors. Typically, receptor-activated K^+ currents desensitize in the sustained presence of agonists to avoid excessive effects on cellular activity. The auxiliary $GABA_B$ receptor subunit KCTD12 induces fast and pronounced desensitization of the K^+ current response. Using proteomic and electrophysiological approaches, we now show that KCTD12-induced desensitization results from a dual interaction with the G protein: constitutive binding stabilizes the heterotrimeric G protein at the receptor, whereas dynamic binding to the receptor-activated Gβγ subunits induces desensitization by uncoupling Gβγ from the effector K^+ channel. While receptor-free KCTD12 desensitizes K^+ currents activated by other GPCRs in vitro, native KCTD12 is exclusively associated with $GABA_B$ receptors. Accordingly, genetic ablation of KCTD12 specifically alters $GABA_B$ responses in the brain. Our results show that $GABA_B$ receptors are endowed with fast and reversible desensitization by harnessing KCTD12 that intercepts Gβγ signaling.

INTRODUCTION

GPCRs and G-protein-regulated ion channels represent fundamental cellular signal transduction systems (Brown and Birnbaumer, 1990; Dascal, 2001; Dunlap et al., 1987; Pierce et al., 2002; Wickman and Clapham, 1995). GPCRs activate heterotrimeric G proteins by catalyzing the exchange of GDP for GTP in Gα, leading to dissociation of Gα·GTP from Gβγ. Released Gα·GTP and Gβγ have independent capacities to regulate effectors such as enzymes and ion channels. Gβγ released from a variety of GPCRs directly gates G-protein-activated inwardly rectifying K^+ (GIRK or Kir3) channels (Betke et al., 2012; Lüscher and Slesinger, 2010) and voltage-activated Ca^{2+} channels (Betke et al., 2012; Tedford and Zamponi, 2006), which influences neuronal activity throughout the brain. Typical examples of such GPCRs are the $GABA_B$ receptors that are activated by GABA, the main inhibitory neurotransmitter in the CNS (Chalifoux and Carter, 2011; Gassmann and Bettler, 2012). Presynaptic $GABA_B$ receptors inhibit voltage-activated Ca^{2+} channels to reduce the release of GABA and other neurotransmitters. Postsynaptic $GABA_B$ receptors activate Kir3 channels and thus inhibit neuronal activity by local shunting or by generating hyperpolarizing postsynaptic potentials. Since $GABA_B$ receptors regulate a wide variety of physiological processes in the nervous system, including neuronal firing, synaptic plasticity, and spontaneous network oscillations, the activity of $GABA_B$ receptors needs to be temporally precise. In the continuous presence of the agonist, $GABA_B$ receptors exhibit a time-dependent decrease in receptor response to avoid prolonged effects on neuronal activity, a phenomenon referred to as desensitization (Cruz et al., 2004; Sickmann and Alzheimer, 2003; Sodickson and Bean, 1996; Wetherington and Lambert, 2002). It is emerging that the desensitization of $GABA_B$ receptor-activated K^+ currents observed in neurons integrates distinct mechanistic underpinnings. First, protein kinases such as PKA or CaMKII regulate desensitization by directly phosphorylating the receptor and influencing its internalization from the cell surface (Couve et al., 2002; Guetg et al., 2010). These phosphorylation-dependent processes typically operate on timescales of minutes to hours. Second, the "regulator of G-protein signaling" protein 4 (RGS4) induces a faster form of desensitization that occurs within seconds of agonist application (Fowler et al., 2007; Mutneja et al., 2005). RGS proteins are "GTPase-activating proteins" (GAPs) that promote desensitization by accelerating the rate of GTP hydrolysis at Gα (Ross and Wilkie, 2000). Third, we recently reported that the K^+ channel tetramerization domain (KCTD)-containing proteins 8, 12, 12b, and 16 represent a novel family of proteins regulating $GABA_B$ receptor-activated K^+ and Ca^{2+} currents (Schwenk et al., 2010). The KCTDs are cytoplasmic proteins that constitutively bind to the C-terminal domain of $GABA_{B2}$ (Ivankova et al., 2013; Schwenk et al., 2010), which together with $GABA_{B1}$ forms obligate heteromeric $GABA_{B(1,2)}$ receptors. All four KCTDs accelerate the rise time

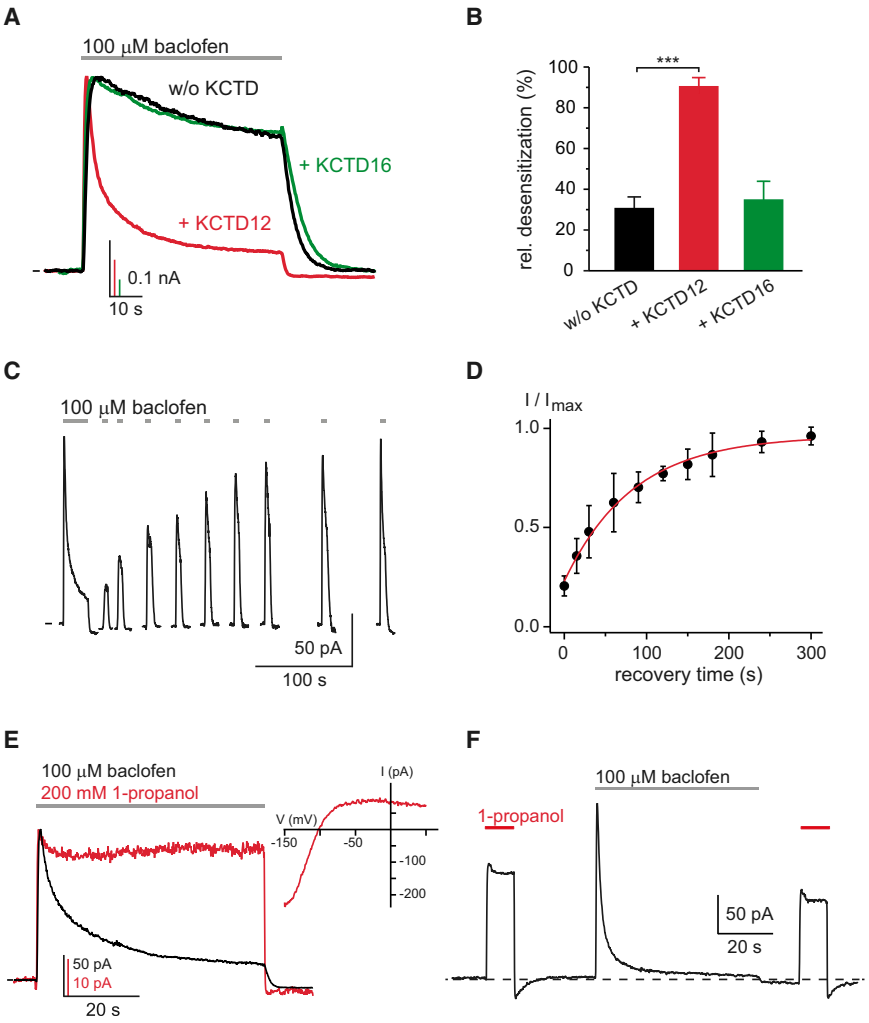

Figure 1. KCTD12-Induced Desensitization Is Activity Dependent, Reversible, and Operates Upstream of Kir3 Channels

(A) Representative traces of K^+ currents activated by baclofen and recorded at −50 mV in CHO cells expressing $GABA_B$ receptors and Kir3.1/3.2 channels either with or without (w/o) KCTD12 or KCTD16. The extracellular K^+ concentration was 2.5 mM; scaling for current and time as indicated. KCTD12 but not KCTD16 induces pronounced and rapid desensitization of the K^+ currents.

(B) Bar graph summarizing the relative desensitization of baclofen-induced K^+ currents. The relative desensitization was calculated as (1 − (ratio of current amplitude after 60 s versus peak current)) × 100. Values are mean ± SD of 60 (w/o KCTD), 84 (KCTD12), and 8 (KCTD16) experiments. ***$p < 0.001$; Dunnett's multiple comparison test.

(C) Recovery of baclofen-activated Kir3 currents from KCTD12-induced desensitization. After an initial 25 s application of baclofen to induce desensitization, baclofen was applied at various time intervals.

(D) Amplitudes (I) of current responses at various time intervals normalized to the initial peak amplitude (I_{max}); data points represented as mean ± SD of 8 experiments. The line represents fit of a monoexponential function to the data with a time constant of 83.6 s.

(E) Representative traces of Kir3 currents activated either by baclofen or 1-propanol and recorded at −50 mV in CHO cells expressing $GABA_B$ receptors, Kir3.1/3.2 channels, and KCTD12. Note that direct activation of Kir3 channels by 1-propanol (red trace) induces largely nondesensitizing currents (14.9% ± 5.1%, n = 10), while activation by baclofen (black trace) induces strongly desensitizing currents (88.8% ± 5.9%, n = 10, $p < 0.001$, paired t test). Inset shows I-V relation determined with a voltage ramp during application of 1-propanol.

(F) Kir3.2 channels are efficiently activated by 1-propanol before and after near complete desensitization of the currents by baclofen. See also Figure S1.

of receptor-activated K^+ currents while only KCTD12 and KCTD12b induce fast and pronounced current desensitization (Schwenk et al., 2010; Seddik et al., 2012). Desensitization is due to the particular H1 homology domain in KCTD12 and KCTD12b as well as the absence of an antagonistic H2 homology domain present in KCTD8 and KCTD16 (Seddik et al., 2012). The mechanism by which the KCTDs regulate $GABA_B$ receptor-activated K^+ and Ca^{2+} currents is unknown.

Here we show that KCTD8, KCTD12, and KCTD16 all constitutively bind to the G protein, which stabilizes the G protein at the receptor and underlies accelerated K^+-current responses. In addition, selectively KCTD12 binds to the activated Gβγ subunits at their interface with Kir3 channels, thereby uncoupling Gβγ from the channels. This postreceptor mechanism of desensitization is fully reversible and rendered receptor-specific through the exclusive association of native KCTD12 protein with $GABA_B$ receptors. Thus, these findings identify a unique receptor-specific mechanism for fast desensitization of G-protein-activated K^+ currents.

RESULTS

KCTD12-Induced Desensitization of $GABA_B$-Activated Kir3 Currents Is Reversible and Operates Upstream of the Channel

To study the desensitization of $GABA_B$ receptor-activated K^+ currents, we performed whole-cell patch-clamp recordings from CHO cells expressing $GABA_B$ receptors and Kir3 channels with or without KCTD proteins. Application of the agonist baclofen to KCTD-free or KCTD16-containing $GABA_B$ receptors elicited robust outward K^+ currents that slightly, and similarly, decreased in amplitude during a 1 min application period (Figures 1A and 1B). In contrast, KCTD12-containing $GABA_B$ receptors elicited K^+ currents that almost completely desensitized (Figures 1A and 1B). The time course of KCTD12-induced desensitization was approximated by a double exponential function with time constants of 1.9 ± 0.3 s (relative contribution to desensitization 42.4% ± 11.2%) and 14.3 ± 2.0 s. The KCTD12-induced desensitization was readily reversible upon removal of

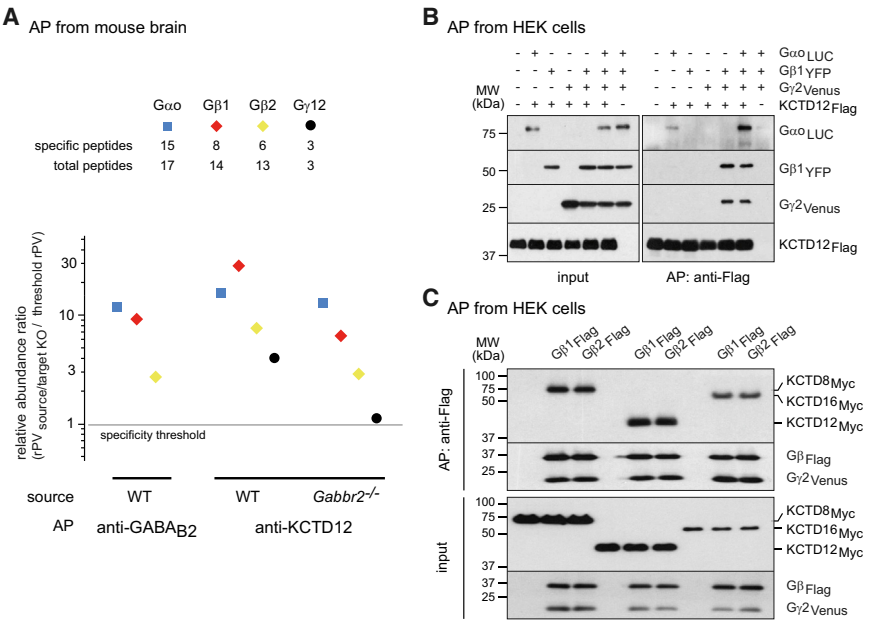

Figure 2. Binding of KCTDs to the G Protein Does Not Require $GABA_B$ Receptors

(A) G protein subunits Gαo, Gβ1, Gβ2, and Gγ12 specifically copurify in APs from WT and *Gabbr2*$^{-/-}$ mouse brain membranes using *anti-KCTD12* or *anti-*$GABA_{B2}$ antibodies. Specificity of the G protein subunit interaction with KCTD12 is determined by the relative abundance of specific peptides in APs versus target knockout controls (rPV) compared to threshold rPV (normalized to 1) (Schwenk et al., 2012). The number of specific and total peptides retrieved by mass spectrometry for any G protein subunit is indicated. Note copurification of G protein subunits in *anti-KCTD12* APs from *Gabbr2*$^{-/-}$ brains in which KCTD12 is not associated with $GABA_B$ receptors.

(B and C) G protein subunits copurify with KCTDs from membranes of transfected HEK293T cells. In (B), the *Renilla* Luciferase-tagged Gαo ($Gαo_{LUC}$), the yellow fluorescent protein-tagged Gβ1 ($Gβ1_{YFP}$), and the Venus-tagged Gγ2 ($Gγ2_{Venus}$) were expressed with or without Flag-tagged KCTD12. In (C), Myc-tagged KCTD8, KCTD12, and KCTD16 and Venus-tagged Gγ2 ($Gγ2_{Venus}$) were expressed with or without the Flag-tagged Gβ isoforms $Gβ1_{Flag}$) or $Gβ2_{Flag}$). APs were performed with *anti-Flag* antibodies and analyzed by western blot with antibodies against *Renilla* Luciferase, Myc, Flag, and GFP. MW, molecular weight; Venus, variant of GFP. See also Figure S2.

baclofen. After near complete baclofen-induced desensitization, the responses to subsequent baclofen applications (Figure 1C) fully recovered with a time constant of 83.6 s (fit to the mean, Figure 1D). To investigate whether KCTD12 directly desensitizes Kir3 channels, we activated the channels in a G-protein-independent manner with 1-propanol (Aryal et al., 2009; Kobayashi et al., 1999; Lewohl et al., 1999). In the presence of KCTD12, 1-propanol induced K^+ currents with negligible desensitization (Figure 1E). Moreover, 1-propanol effectively, and similarly, activated Kir3 channels both before and after complete KCTD12-induced current desensitization obtained by applying baclofen for 60 s (Figure 1F). Likewise, 1-propanol still activated Kir3 channels during baclofen-evoked KCTD12-induced current desensitization (Figure S1 available online). Together, these results demonstrate that KCTD12-induced desensitization is fast, fully reversible, activity dependent, and operates upstream of Kir3 channels.

KCTDs Interact with G Protein Subunits

The above results suggest that KCTD12 induces desensitization at the receptor and/or the G protein. We used a proteomic approach combining antibody-based affinity purifications (APs) with high-resolution quantitative mass spectrometry (Müller et al., 2010; Schwenk et al., 2012) to address whether G protein subunits directly interact with KCTD12 in native tissue. For APs, we equilibrated the entire pool of solubilized KCTD12 protein in mouse brain membranes with *anti-KCTD12* antibodies. To control the specificity of the APs, we used membrane fractions from *Kctd12* knockout (*Kctd12*$^{-/-}$) mice (Metz et al., 2011) (target KO; Figure 2A). The *anti-KCTD12* antibody copurified $GABA_{B1}$, $GABA_{B2}$ (but no other GPCRs) and the G protein subunits Gαo, Gβ1, Gβ2, and Gγ12 (Figure 2A). Copurification of the G protein subunits was also observed when KCTD12 was not associated with $GABA_B$ receptors (using *Gabbr2*$^{-/-}$ mice [Gassmann et al., 2004] for APs; Figure 2A). This suggests that KCTD12 directly interacts with G proteins.

Interactions of KCTD12 with G proteins were confirmed in APs from HEK293T cells coexpressing combinations of epitope-tagged KCTD proteins and G protein subunits. FLAG-tagged KCTD12 copurified the G protein either as a Gαβγ trimer or as a Gβγ dimer (Figure 2B). Notably, copurification of individual G protein subunits with KCTD12 either failed (Gβ, Gγ) or was very inefficient (Gαo). APs with purified recombinant KCTD12 and Gβ1γ2 proteins confirmed that these proteins directly interact with each other (Figure S2). These results identify the Gβγ dimer as the primary interaction partner of KCTD12 (Figure 2B). Experiments with KCTD8 and KCTD16 confirmed that all KCTD subunits of $GABA_B$ receptors bind to Gβγ (Figure 2C).

KCTD12 Dynamically Binds Activated Gβγ Subunits and Prevents Their Interaction with Kir3 Channels

We next tested in transfected CHO cells whether KCTD12 desensitizes K^+ currents by directly acting at the G protein. For this purpose, we activated Kir3 channels in a receptor-independent manner with the nonhydrolysable GTP-analog guanosine 5′-O-(3-thiotriphosphate) (GTPγS), which we perfused into the cell via the recording pipette (Figure 3A). By exchanging for GDP at Gα, GTPγS liberates Gβγ and constitutively activates Kir3 channels (Breitwieser and Szabo, 1988; Dunlap et al., 1987; Gilman, 1987; Kurachi et al., 1987; Leaney et al., 2004; Logothetis et al., 1987; Stryer and Bourne, 1986). In the absence

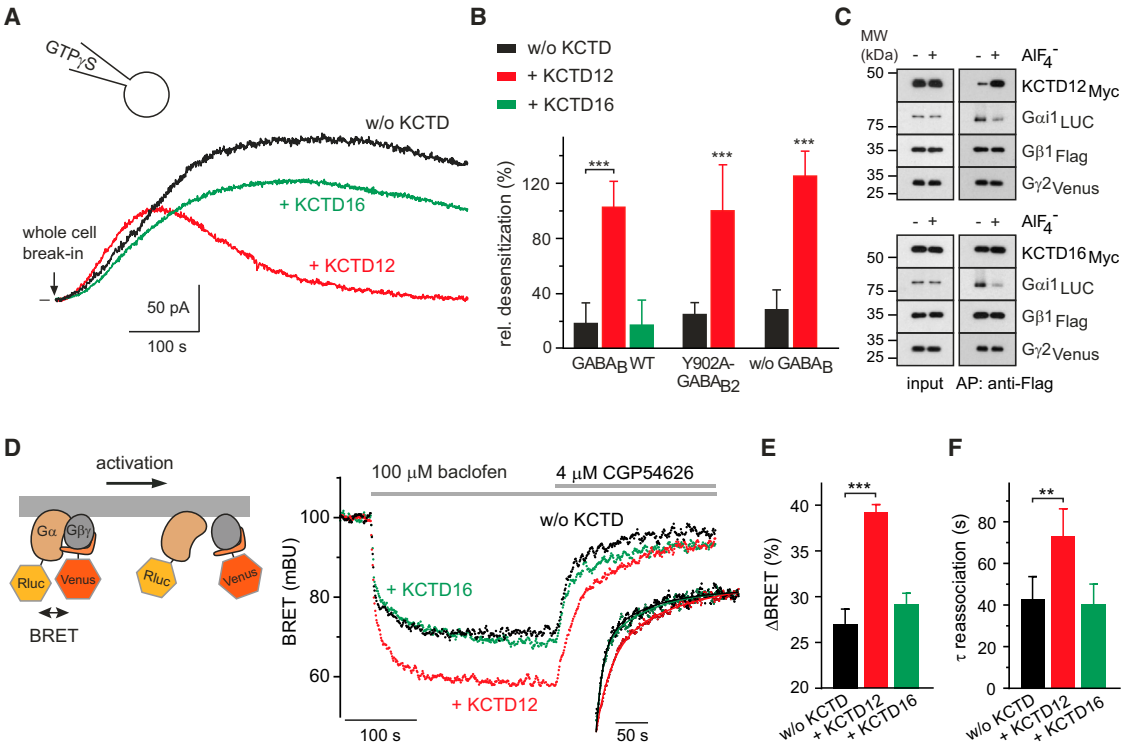

Figure 3. KCTD12-Induced Desensitization Requires Interaction with the Activated G Protein

(A) Representative Kir3 currents activated by intracellular perfusion of GTPγS (0.6 mM) and recorded at −50 mV in transfected CHO cells expressing Kir3.1/3.2 channels with or without (w/o) KCTD12 or KCTD16. Note that KCTD12-induced desensitization only occurs after activation of the G protein by GTPγS and that channel activation by Gβγ is faster than desensitization by KCTD12. Due to the competition of both processes, the peak current is reached earlier and reduced in amplitude compared to control (KCTD12: 62.8 ± 61.9 pA, n = 13; w/o KCTD12: 128.0 ± 98.8 pA, n = 28; p = 0.025, Student's t test). KCTD16 fails to desensitize the current response.

(B) Bar graph summarizing the desensitization of GTPγS-induced responses. The relative desensitization (calculated after 10 min of GTPγS perfusion) was similar in CHO cells expressing KCTD12 without $GABA_B$ receptors (w/o $GABA_B$) or together with WT $GABA_B$ receptors or mutant Y902A-$GABA_{B2}$ receptors that do not associate with the KCTDs (Schwenk et al., 2010). Data are represented as mean ± SD of 28 (WT $GABA_B$, w/o KCTD), 13 (WT $GABA_B$, + KCTD12), 11 (WT $GABA_B$, + KCTD16), 9 (Y902A-$GABA_{B2}$, w/o KCTD), 7 (Y902A-$GABA_{B2}$, + KCTD12), 10 (w/o $GABA_B$, w/o KCTD), and 6 (w/o $GABA_B$, + KCTD12) recordings. ***p < 0.001; Dunnett's multiple comparison test and Student's t test.

(C) Constitutive activation of the G protein with AlF_4^- exposes the activity-dependent binding site on Gβγ and selectively increases KCTD12 binding to Gβγ. HEK293T cells, expressing Gαi1$_{LUC}$, Gβ1$_{Flag}$, Gγ2$_{Venus}$, and either KCTD12$_{Myc}$ or KCTD16$_{Myc}$ were lysed in the absence or presence of AlF_4^-. APs were performed with *anti-Flag* antibodies and analyzed by western blot with antibodies against *Renilla* Luciferase, Myc, Flag, and GFP.

(D) Baclofen-induced changes in the BRET ratio determined in CHO cells expressing $GABA_B$ receptors, Gαo-*R*Luc, Flag-Gβ2, and Venus-Gγ2 without (w/o) or with KCTD12 and KCTD16. Single experiments carried out in parallel are shown. Scheme on the left indicates conformational changes during G protein activation that are monitored by BRET measurements. The BRET ratio decreases during G protein activation due to conformational rearrangement of the Gαo-*R*Luc and Venus-Gγ2 subunits. After receptor blockade with the antagonist CGP54626, reassociation of the G protein is significantly slowed in the presence of KCTD12. The BRET recovery phases are shown fitted to a double exponential function (inset).

(E) Bar graph of the changes in BRET ratio determined in experiments as in (D).

(F) Bar graph of the amplitude-weighted mean time constants obtained by fitting the BRET recovery phase to a double exponential function in experiments as in (D). Data in (E) and (F) are represented as mean ± SD of five experiments. ***p < 0.001; **p < 0.01; Kruskal Wallis test.

of KCTDs or in the presence of KCTD16, GTPγS induced slowly rising inwardly rectifying K^+ currents that exhibited modest desensitization over the 10 min recording period (Figures 3A and 3B). In contrast, in the presence of KCTD12 the currents exhibited pronounced desensitization eventually leading to a decrease in amplitudes close to baseline (Figures 3A and 3B). Similar results for KCTD12-induced desensitization were obtained when Kir3 channels were activated by GTPγS in the presence of either wild-type (WT) $GABA_B$ receptors or mutant Y902A-$GABA_{B2}$ receptors that are unable to bind KCTD12 (Correale et al., 2013; Schwenk et al., 2010) (Figure 3B). These results demonstrate that KCTD12-induced Kir3 current desensitization requires activation of the G protein but does not require assembly of KCTD12 with $GABA_B$ receptors. Moreover, since GTPγS is nonhydrolysable, these experiments show that KCTD12 does not promote desensitization through GAP activity at Gα (Mutneja et al., 2005; Ross and Wilkie, 2000). Rather, the results point to an activity-dependent interaction of KCTD12 with the G protein, in addition to the constitutive interaction that KCTD12 shares with KCTD8 and KCTD16 (Figure 2). Indeed, constitutive

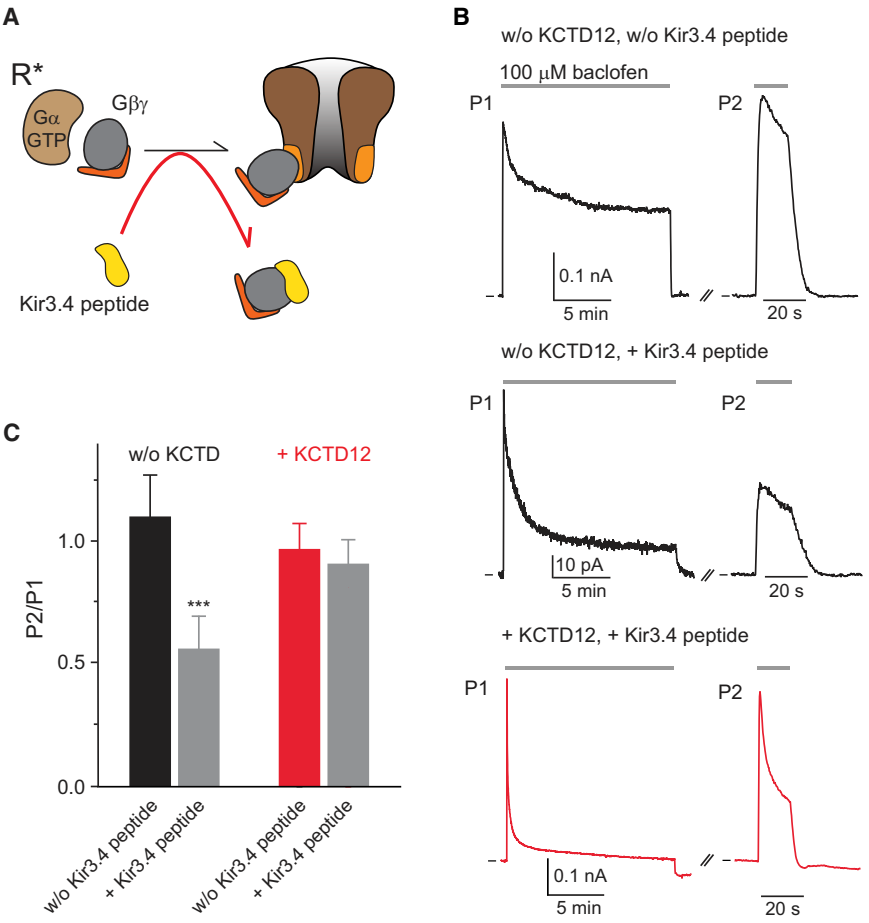

Figure 4. KCTD12 Occludes the Kir3 Interaction Site on Gβγ

(A) Scheme illustrating activity-dependent interference with Gβγ binding to Kir3 channels using a Kir3.4-derived peptide.

(B) Representative traces of Kir3 currents evoked by two consecutive baclofen applications (first application was for 15 min, second application for 15 s after a 7 min interval) to CHO cells expressing $GABA_B$ receptors in the absence (top, middle) or presence (bottom) of KCTD12. Cells were perfused with control intracellular solution (w/o Kir3.4 peptide) or with intracellular solution supplemented with 40 μM Kir3.4-peptide (+ Kir3.4 peptide).

(C) Bar graphs summarizing the amplitude ratios of peak K^+ currents recorded during the second (P2) and first (P1) baclofen application. Data are represented as mean ± SD of 9 (w/o KCTD, w/o Kir3.4 peptide), 8 (w/o KCTD, + Kir3.4 peptide), 5 (+ KCTD12, w/o Kir3.4 peptide), and 6 (+ KCTD12, + Kir3.4 peptide) experiments. ***$p < 0.001$; Student's t test. Note that KCTD12 counteracts the reduction of the peak amplitude by the Kir3.4-peptide during the second baclofen application. See also Figure S3.

activation of the G protein with AlF_4^- selectively increased KCTD12 binding to Gβγ, consistent with an activity-dependent binding site on Gβγ that is unique to KCTD12 (Figure 3C).

We next studied whether KCTD12 and KCTD16 differentially influence G protein conformational rearrangements during G protein activation, using bioluminescence resonance energy transfer (BRET) experiments in transfected CHO cells (Digby et al., 2006; Frank et al., 2005) (Figures 3D and 3E). Indeed, KCTD12 but not KCTD16 induced a significant increase in the magnitude of the BRET change during G protein activation. Moreover, reassociation of the G protein was slowed in the presence of KCTD12 (Figures 3D and 3F). Altogether, the data are compatible with KCTD12 influencing conformational changes of the G protein and/or increasing steady-state G protein dissociation in an activity-dependent manner.

With native $GABA_B$ receptors where $GABA_{B(1,2)}$, KCTD12, and the G protein reside in close proximity (Schwenk et al., 2010), constitutive and activity-dependent binding of KCTD12 to Gβγ may be envisaged as follows: receptor activation of the G protein, which is stabilized at the receptor via constitutive binding to KCTD12, promotes both activation of Kir3 channels and activity-dependent interaction of KCTD12 with Gβγ. Competition between KCTD12 and Kir3 channels for Gβγ reduces steady-state Gβγ interaction with the channels, which desensitizes the current response. Finally, the Gα·GDP subunit displaces KCTD12 from its activity-dependent binding site on Gβγ and reconstitutes the heterotrimeric G protein, which renders the desensitization mechanism reversible. To address whether KCTD12 occludes the channel binding site of Gβγ (Ford et al., 1998; Whorton and MacKinnon, 2013), we used a peptide derived from the Gβγ binding site of the Kir3.4 protein (Figure 4A). This Kir3.4-peptide inhibits Gβγ activation of Kir3 channels with an IC_{50} of 0.6 μM (Krapivinsky et al., 1998). Perfusion of the Kir3.4-peptide (40 μM) into CHO cells expressing $GABA_B$ receptors and Kir3 channels resulted in strong desensitization of the K^+ currents during a 15 min baclofen application (Figure 4B, middle). The peptide-induced desensitization was slowly reversible; a 7 min period showed recovery of the baclofen response to about half of the initial peak current amplitude (Figures 4B, middle, and 4C), while complete recovery was obtained in the absence of the peptide (Figures 4B, top, and 4C). In the combined presence of KCTD12 and Kir3.4-peptide, the desensitization was faster than with the peptide alone (Figure 4B, bottom). However, a close to complete recovery of the peak K^+ current amplitude was obtained within the 7 min period, showing that KCTD12 significantly counteracted the lasting inhibitory effect of the Kir3.4-peptide (Figures 4B, bottom, and 4C). The most likely explanation of these data is that KCTD12 efficiently competes with the Kir3.4-peptide for binding to activated Gβγ. While allosteric effects of KCTD12 on Kir3.4-peptide binding cannot be fully ruled out, we consider this possibility less likely. The Gβγ dimer is assumed to be a relatively rigid scaffold for protein binding and its X-ray structure remains unperturbed when bound to various peptides or effectors (Lin and Smrcka, 2011;

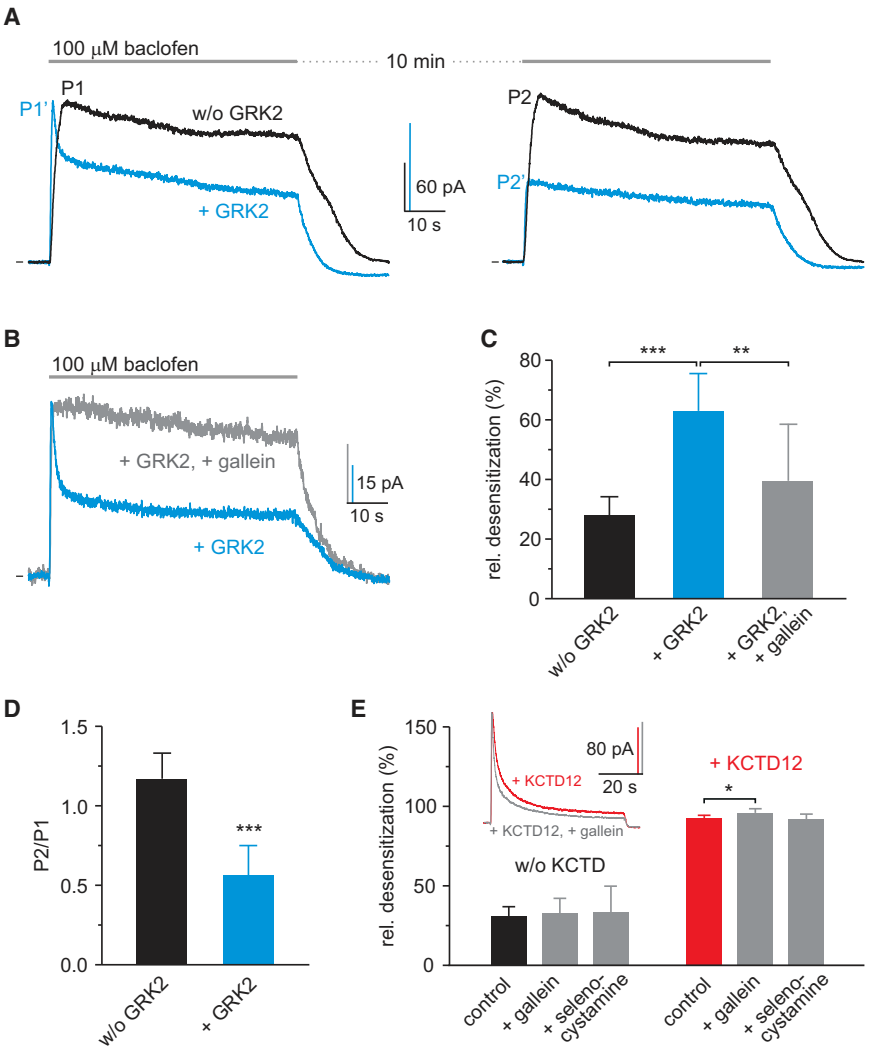

Figure 5. KCTD12 and GRK2 Differ in Their Binding to Gβγ and in the Reversibility of Desensitization

(A) Representative traces of Kir3 currents evoked by two consecutive baclofen applications in an interval of 10 min to CHO cells expressing $GABA_B$ receptors with or without (w/o) GRK2.

(B) Traces of Kir3 currents recorded from GRK2-expressing cells in the absence (blue) or presence (gray) of 20 μM gallein.

(C) Bar graphs summarizing Kir3 current desensitization. Data are mean ± SD of 7 (w/o GRK2), 11 (+ GRK2), and 6 (+GRK2, + gallein) experiments. ***p < 0.001; **p < 0.01; Dunnett's multiple comparison test or Student's t test.

(D) Bar graph summarizing the amplitude ratios of peak K^+ currents recorded during the second (P2) and first (P1) baclofen application (A). Data are represented as mean ± SD of 5 (w/o GRK2) and 9 (+ GRK2) recordings. ***p < 0.001; Dunnett's multiple comparison test. Note that GRK2-induced desensitization does not revert during the 10 min period.

(E) Bar graph summarizing the effects of 20 μM gallein and 20 μM selenocystamine on Kir3 current desensitization in CHO cells with or without KCTD12. Data are represented as mean ± SD of 8 (control, w/o KCTD), 18 (+ gallein, w/o KCTD), 10 (selenocystamine, w/o KCTD), 7 (control, + KCTD12), 15 (+ gallein, + KCTD12), and 6 (+ selenocystamine, + KCTD12) recordings. *p < 0.05; Dunnett's multiple comparison test. Inset shows representative traces of baclofen-evoked Kir3 currents recorded from KCTD12-expressing cells in the absence (+ KCTD12) or presence of gallein (+ KCTD12, + gallein). See also Figure S4.

Oldham and Hamm, 2006). Moreover, in our experiments, KCTD12 was unable to displace the Kir3.4 peptide once bound to Gβγ (Figure S3).

Similar to KCTD12, GPCR kinase 2 (GRK2) binds to Gβγ and induces fast desensitization of GPCR-activated Kir3 currents in heterologous cells (Raveh et al., 2010). Because KCTD12 and GRK2 share no sequence or structural similarity, we compared the properties of the desensitization induced by the two proteins. Expression of GRK2 in CHO cells increased desensitization of baclofen-activated Kir3 currents by ~30% (Figures 5A and 5C). In contrast to KCTD12-induced desensitization, the GRK2-induced desensitization was irreversible within a 10 min period (Figures 5A and 5D). Moreover, the GRK2-induced desensitization was prevented by gallein (Figures 5B and 5C), a compound binding with high-affinity to the protein-protein interaction "hot spot" of Gβγ (Lehmann et al., 2008; Scott et al., 2001). Gallein and selenocystamine (Dessal et al., 2011), a structurally unrelated compound that also binds to the "hot spot," did not prevent KCTD12-induced desensitization (Figure 5E). Thus, KCTD12 and GRK2 binding differs in its sensitivity to compounds that bind to the "hot spot" of Gβ. Nonetheless, as GRK2 and KCTD12 can displace each other from Gβγ (Figure S4), the binding domains of the two proteins overlap, probably in the channel-binding area on Gβγ.

KCTD12-Induced Desensitization Is Specific for $GABA_B$ Receptors

If KCTD12 induces desensitization by acting at Gβγ, it should not only desensitize $GABA_B$-activated Kir3 currents but also those activated by other GPCRs, as long as there is free KCTD12 available to bind to G proteins. Indeed, we observed KCTD12-induced desensitization in heterologous cells with various GPCRs. For example, activation of adenosine A1 (Figure 6A) or mGlu2 receptors (Figures S5A and S5C) in the presence of KCTD12 in transfected CHO cells yielded desensitizing Kir3 currents. Activation of mutant Y902A-$GABA_{B2}$ receptors that cannot bind KCTD12 gave rise to strongly desensitizing Kir3 currents in *Xenopus* oocytes only after injection of KCTD12 cRNA in large excess over $GABA_B$ receptor cRNA (ratio of 32:1; Figure 6B). Under these conditions, KCTD12 levels are sufficiently high to decrease basal currents (induced by endogenous or overexpressed exogenous Gβγ; Figures S5F–S5H) and to desensitize Y902A-$GABA_{B2}$

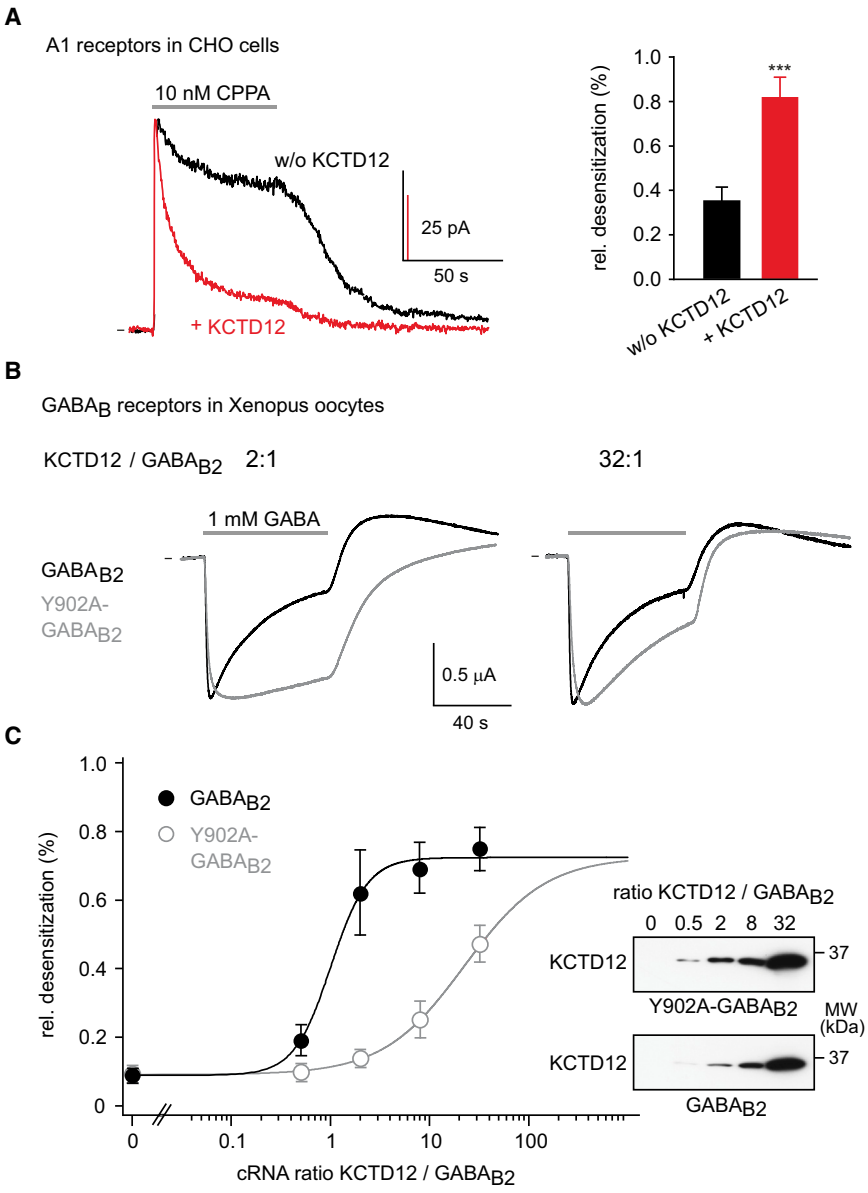

Figure 6. Receptor Specificity of KCTD12-Induced Desensitization Depends on the KCTD12/GABA$_B$ Ratio

(A) Left: representative K^+ current responses elicited by 2-chloro-N6-cyclopentyladenosine (CCPA) in CHO cells expressing adenosine A1 receptors with (+ KCTD12) or without (w/o KCTD12) KCTD12. Right: bar graph summarizing desensitization of CCPA-induced K^+ currents; data are represented as mean ± SD of 5 experiments.

(B) GABA-activated Kir3 currents recorded at −50 mV from *Xenopus* oocytes injected with cRNA encoding KCTD12 and either WT GABA$_{B2}$ or mutant Y902A-GABA$_{B2}$ at the indicated ratios. Note that mutant receptors only produced desensitizing responses with a high expression level of KCTD12, while WT receptors produced desensitizing responses already at a low KCTD12 expression level. Y902A-GABA$_{B2}$ cRNA expresses slightly less protein than WT GABA$_{B2}$ cRNA (Figure S5I), showing that the difference in desensitization is not due to higher expression of Y902A-GABA$_{B2}$ than WT GABA$_{B2}$.

(C) Relative desensitization of K^+ currents in *Xenopus* oocytes at different cRNA ratios for KCTD12 and WT GABA$_{B2}$ or Y902A-GABA$_{B2}$. Data points are represented as mean ± SD of 6–11 experiments. Lines are the results of a Hill equation fitted to the data yielding values for half-maximal effect and slope factor of 1.01 and 2.34 for WT GABA$_{B2}$ and 22.5 and 1.06 for Y902A-GABA$_{B2}$. Inset: western blots showing expression of KCTD12 protein in oocytes injected with the indicated cRNA ratios. See also Figure S5.

receptor-activated Kir3 currents (Figures 6B and 6C). At equimolar amounts of injected KCTD12 and GABA$_B$ receptor cRNA, activation of Y902A-GABA$_{B2}$ receptors elicited robust K^+ currents that, however, failed to desensitize. This contrasts with WT GABA$_B$ receptors, which exhibited strongly desensitizing Kir3 currents already at low cRNA ratios (Figures 6B and 6C). Dose response relations for KCTD12/GABA$_{B2}$ cRNA ratios versus Kir3 current desensitization revealed a more than 10-fold difference between WT and mutant receptors (Figure 6C). Together, these results suggest that WT GABA$_B$ receptors promote desensitization by capturing KCTD12, even at low expression levels, and juxtaposing it to the activated G protein. Accordingly, transfer of the KCTD-binding domain of GABA$_{B2}$ to a metabotropic glutamate receptor (mGlu2-GABA$_{B2}$-CT) endowed this chimera with rapid and pronounced KCTD12-induced desensitization, similar to that of GABA$_B$ receptors (Figures S5A–S5E).

The above results show that the relative amounts of GABA$_B$ receptors and KCTD12 will determine the receptor specificity of desensitization. Next, we therefore examined to what extent KCTD12 protein is associated with GABA$_B$ receptors in the adult mouse brain. For this purpose, we solubilized the complete pool of KCTD12 protein present in brain membrane fractions and separated the solubilized proteins by native gel electrophoresis (BN-PAGE) and SDS-PAGE. Western blots of WT brain probed with *anti-KCTD12* and *anti-GABA$_{B2}$* antibodies demonstrated that the vast majority of KCTD12 protein is assembled into high-molecular weight GABA$_B$ receptor complexes (Figure 7A, top). The western blot in Figure 7A (enlarged at shorter exposure time in the inset in Figure 7B) further indicates that KCTD12 only assembles into a fraction of GABA$_B$ receptors with an apparent molecular mass of ~0.6 MDa. A minor fraction of KCTD12 protein, possibly dissociated from GABA$_{B2}$ during solubilization, focused in the mass range of 0.15 to 0.18 MDa, the expected value for assemblies of KCTD12 tetramers (Schwenk et al., 2010) and Gβγ. When using membrane fractions from *Gabbr2*$^{-/-}$ mice, the entire pool of KCTD12 protein appeared at the lower mass range (Figure 7A, bottom).

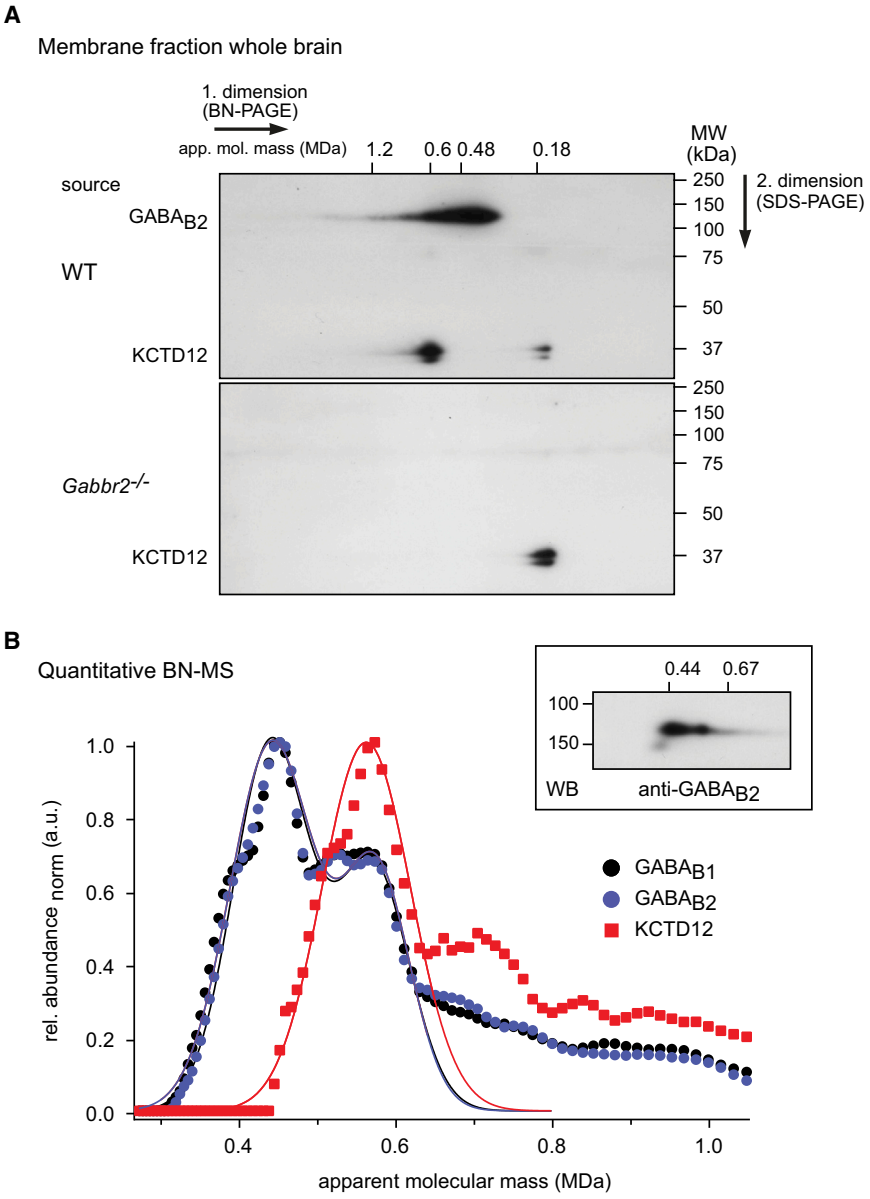

Figure 7. KCTD12 in the Adult Mouse Brain Is Exclusively Associated with $GABA_B$ Receptors

(A) Two-dimensional gel separations of native $GABA_B$ receptors in membrane fractions prepared from whole brains of WT and *Gabbr2*$^{-/-}$ mice. $GABA_{B2}$ and KCTD12 were revealed on western blots. Apparent molecular mass (BN-PAGE) and molecular weight (SDS-PAGE) are as indicated. Note that KCTD12 coassembles into high-molecular weight $GABA_B$ receptor complexes in WT but not in *Gabbr2*$^{-/-}$ mice. The total amounts of KCTD12 protein in *Gabbr2*$^{-/-}$ and WT mice do not differ significantly (Figure S6).

(B) Abundance-mass profiles determined by BN-MS analyses for solubilized $GABA_{B1}$, $GABA_{B2}$, and KCTD12. Each data point represents the amount determined for the respective protein in one gel slice (total of 95 slices) normalized to maximum; symbols are as indicated. Lines are the result of a mono (red) or double component (blue) Gaussian function fitted to the data with values for apparent complex mass peaks of 0.56 MDa (KCTD12), 0.44 MDa, and 0.57 MDa ($GABA_{B2}$). Note tight coassembly of KCTD12 and a fraction of $GABA_B$ receptors causing a shift in apparent molecular mass consistent with the weight of KCTD12-Gβγ assemblies. Inset shows western blot of the 2D-PAGE-separated $GABA_B$ receptors from (A) exposed for a shorter period to resolve the two populations of $GABA_B$ receptors. See also Figure S6.

Thus, in the adult mouse brain, KCTD12 almost exclusively associates with $GABA_B$ receptors. This agrees with the results from *anti-KCTD12* APs that failed to identify additional GPCRs associating with KCTD12 (see above). We further investigated the $GABA_B$-KCTD12 assembly by combined use of BN-PAGE and quantitative high-resolution mass spectrometry (BN-MS [Schwenk et al., 2012]). BN-PAGE slices (400 μm) containing $GABA_B$ receptors from whole-brain preparations were individually analyzed for the relative molecular abundance of $GABA_{B1}$, $GABA_{B2}$, and KCTD12 proteins. The abundance profiles generated from 95 consecutive slices showed precise coincidence for $GABA_{B1}$ and $GABA_{B2}$ and identified two major populations of $GABA_{B(1,2)}$ receptors in the apparent molecular mass range of 0.35 to 0.7 MDa (Figure 7B): one population representing coassemblies with KCTD12 had a mass of ~0.57 MDa, the other population had a mass of ~0.44 MDa and is devoid of KCTD12 (and other KCTDs, data not shown; Figure 7A). Fitting Gaussian distributions to these two populations showed that 40% of $GABA_B$ receptors are assembled with KCTD12, while 60% are free of KCTDs (Figure 7B). BN-PAGE analysis therefore supports that in the adult brain KCTD12 exclusively associates with a fraction of $GABA_B$ receptors. This indicates that $GABA_B$ receptors are present in excess of KCTD12 and implies that KCTD12-induced desensitization is highly $GABA_B$ receptor specific.

Altered $GABA_B$ Receptor-Activated K^+ Currents in KCTD Knockout Mice

We previously reported that overexpression of KCTD12 in cultured hippocampal neurons strongly desensitizes baclofen-induced K^+ currents (Schwenk et al., 2010). We now addressed whether loss of the KCTDs in hippocampal neurons of *Kctd12*$^{-/-}$ (Metz et al., 2011) and *Kctd8/12/16* triple knockout (*Kctd8/12/16*$^{-/-}$) mice (Metz et al., 2011) reduces desensitization of baclofen-induced K^+ currents. Of note, KCTD12b is selectively expressed in the medial habenula and therefore has no effect in the hippocampus (Metz et al., 2011; Schwenk et al., 2010). Indeed, baclofen-induced K^+ currents desensitized significantly

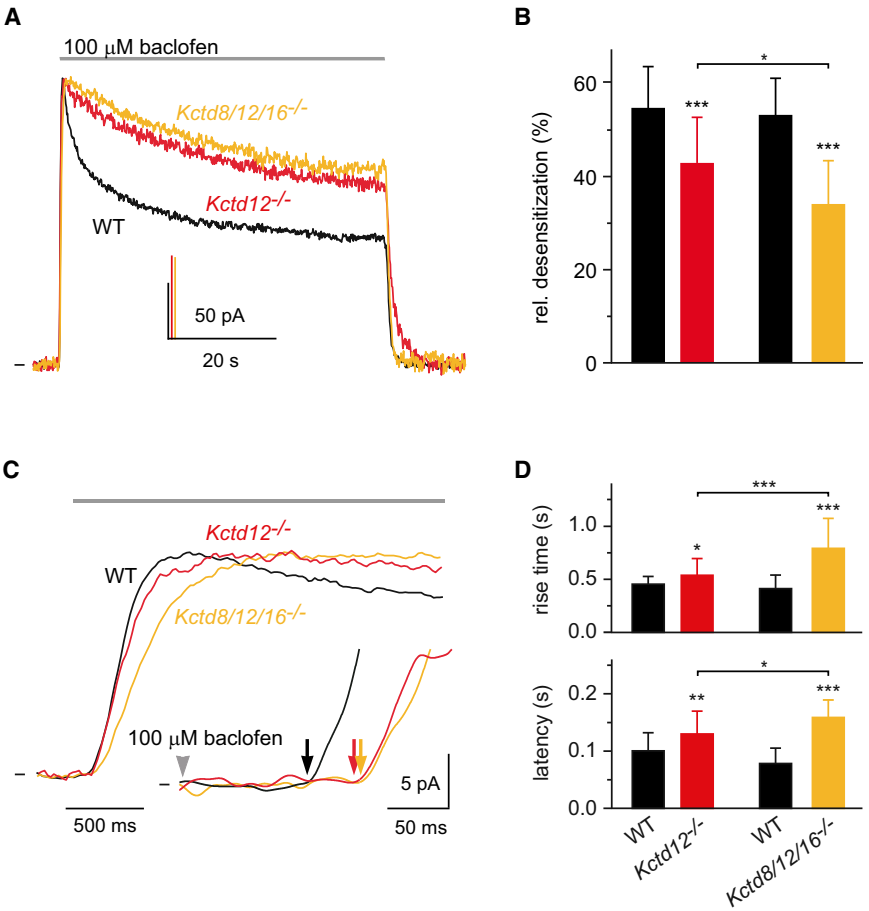

Figure 8. Altered $GABA_B$ Receptor-Activated K^+ Currents in *Kctd12*$^{-/-}$ and *Kctd8/12/16*$^{-/-}$ Mice

(A) Representative traces of baclofen-evoked K^+ currents recorded from cultured hippocampal neurons of *Kctd12*$^{-/-}$ (red), *Kctd8/12/16* triple knockout (orange), and WT (black) mice.

(B) Bar graph summarizing the desensitization observed in neurons of *Kctd12*$^{-/-}$ (red), *Kctd8/12/16*$^{-/-}$ (orange), and control WT (black) mice. Data are represented as mean ± SD of 31 (*Kctd12*$^{-/-}$), 23 (WT control of *Kctd12*$^{-/-}$), 13 (*Kctd8/12/16*$^{-/-}$), and 17 (WT control of *Kctd8/12/16*$^{-/-}$) neurons. ***p < 0.001; *p < 0.05; Student's t test. *Kctd8/12/16*$^{-/-}$ neurons exhibit a more pronounced reduction in desensitization than *Kctd12*$^{-/-}$ neurons. This may relate to a generally slower G protein/effector channel coupling (C and D). This may reduce the basal desensitization, which is KCTD12 independent and determined by the kinetic properties of the G protein cycle (Chuang et al., 1998; Leaney et al., 2004; Sickmann and Alzheimer, 2003).

(C) Representative traces of baclofen-evoked K^+ currents recorded from neurons of mutant and WT control mice. Inset shows currents at enlarged timescale showing the latency period between start of agonist application (arrow head) and current onset (arrows).

(D) Bar graphs summarizing 5%–95% rise time and latency observed in neurons of mutant and WT mice (neurons as in B). Data are represented as mean ± SD. ***p < 0.001; **p < 0.01; *p < 0.05, Student's t test. See also Figure S7.

less in cultured hippocampal neurons of *Kctd12*$^{-/-}$ and *Kctd8/12/16*$^{-/-}$ mice when compared to neurons of WT mice (Figures 8A and 8B). In contrast, adenosine A1 receptor-induced K^+ currents desensitized to a similar extent in *Kctd12*$^{-/-}$ and WT (*Kctd12*$^{-/-}$: 9.3% ± 3.0%, n = 9; WT: 12.1% ± 4.1%, n = 9; p = 0.124) neurons, consistent with KCTD12 selectively influencing $GABA_B$ receptor signaling. Interestingly, adenosine A1 receptor-induced K^+ currents recorded from *Gabbr2*$^{-/-}$ neurons showed increased desensitization compared to WT neurons (*Gabbr2*$^{-/-}$: 25.5% ± 8.4%, n = 13; WT, 10.2% ± 4.7%, n = 6, p < 0.001). Thus, in the absence of $GABA_B$ receptors, KCTD12 is "released" in amounts that promiscuously regulate Gβγ signaling of other GPCRs, similar to the results obtained with heterologous expression of large amounts of KCTD12 (Figures 6).

All KCTDs shorten the rise time of baclofen-induced K^+ currents in heterologous cells (Schwenk et al., 2010). Moreover, we found that the latency between agonist application and onset of the K^+ current response is significantly shorter in the presence of KCTDs and dependent on KCTD binding to the receptor (Figure S7). Accordingly, the rise times of the baclofen-induced K^+ currents recorded in *Kctd12*$^{-/-}$ and *Kctd8/12/16*$^{-/-}$ neurons were significantly longer than those obtained in WT neurons (Figures 8C and 8D). Of note, *Kctd8/12/16*$^{-/-}$ neurons exhibit a significantly slower rise time than *Kctd12*$^{-/-}$ neurons, consistent with all three KCTDs contributing to a shortening of the rise time (Schwenk et al., 2010). In addition, the latency of the current response was significantly longer in *Kctd12*$^{-/-}$ and *Kctd8/12/16*$^{-/-}$ neurons than in WT neurons (Figures 8C and 8D). Again, the latency was longer in *Kctd8/12/16*$^{-/-}$ than in *Kctd12*$^{-/-}$ neurons, consistent with heterologous data showing that multiple KCTDs can accelerate the onset of the current response (Figure S7). Native KCTDs therefore promote rapid G protein signaling with faster rise times and shorter latency of the receptor response. Accelerated G protein signaling probably relates to the constitutive binding of the G protein to KCTD8, KCTD12, and KCTD16, which stabilizes the G protein at the receptor.

DISCUSSION

This work presents a molecular mechanism for fast and reversible desensitization of G-protein-mediated K^+ current responses. We show that KCTD12 dynamically interacts with Gβγ released from the activated G protein and thus directly competes with Gβγ binding to the effector Kir3 channel. Albeit KCTD12 has the intrinsic ability to inhibit Kir3 currents activated by numerous GPCRs, the exclusive assembly of KCTD12 into $GABA_B$ receptors in the brain results in a highly receptor-specific current desensitization. Activity-dependent interaction with Gβγ is unique to KCTD12. However, KCTD8, KCTD12, and KCTD16 are all able to constitutively bind the G protein through

Gβγ. Constitutive binding of the KCTDs to both the G protein and the receptor appears to stabilize the G protein at the receptor and to accelerate K^+ current responses.

A Reversible Mechanism for Fast Desensitization of $GABA_B$ Receptor Responses

The desensitization of GPCR-activated K^+ currents that is observed within seconds of agonist exposure (Sickmann and Alzheimer, 2002, 2003) is too fast to be explained by classical mechanisms of desensitization, which typically involve receptor phosphorylation, uncoupling of the G protein from the receptor, receptor internalization, and degradation (Evron et al., 2012; Tsao and von Zastrow, 2000). It emerges that fast desensitization is primarily regulated at the postreceptor level. To some extent, fast desensitization is determined by the kinetic properties of the G protein cycle, such as the rates of GDP-GTP exchange and GTP hydrolysis at Gα (Chuang et al., 1998; Leaney et al., 2004; Sickmann and Alzheimer, 2003). Accordingly, fast desensitization is promoted by several proteins acting at the G protein. These proteins include RGS proteins, which increase GTPase activity at Gα (Bender et al., 2004; Chuang et al., 1998; Jeong and Ikeda, 2001; Mutneja et al., 2005; Ross and Wilkie, 2000), and KCTD12 that shields the Kir3 binding site of Gβγ. Moreover, GRK2 was shown to nonenzymatically induce fast desensitization of Kir3 currents by scavenging free Gβγ (Raveh et al., 2010). While both KCTD12 and GRK2 induce desensitization by binding to Gβγ, the respective desensitization mechanisms are profoundly different. KCTD12-induced K^+ current desensitization is fully reversible, whereas GRK2-induced desensitization displays poor reversibility (Figure 5). We found that compounds that bind in the "hot spot" region of Gβ (Dessal et al., 2011; Lehmann et al., 2008) prevent GRK2-, but not KCTD12-, induced desensitization (Figure 5). This is consistent with GRK2 binding at the interface of Gβγ with Gα (Lodowski et al., 2003; Tesmer et al., 2005), which sequesters the heterotrimeric G protein subunits (Tesmer et al., 2005). Moreover, GRK2 probably scavenges free Gβγ away from channels (Raveh et al., 2010), which may contribute to the poor reversibility of the desensitization mechanism. GRK2, by increasing the refractory period of the G protein, appears to be better suited to induce fast and long-lasting desensitization. In contrast, KCTD12, by avoiding the "hot spot" and specifically targeting the channel binding site of Gβγ, allows for fast desensitization and recovery of the receptor response.

Receptor Specificity of KCTD12-Induced Desensitization

Since proteins promoting fast desensitization act at the postreceptor level, fast desensitization is generally expected to lack receptor specificity. Indeed, the mechanism of KCTD12-induced desensitization is not intrinsically receptor specific, as shown in heterologous expression experiments (Figures 6 and S5). Nevertheless, native KCTD12-induced desensitization is exquisitely $GABA_B$ receptor specific. Quantitative proteomic analysis indicates that native KCTD12 is exclusively associated with $GABA_B$ receptors, as no other GPCRs were detected in *anti-KCTD12* APs and genetic lack of $GABA_{B2}$ abolished the appearance of KCTD12 in high-molecular weight protein complexes (Figure 7).

There may be developmental windows, regional cell types, or pathological conditions where KCTD12 is expressed in excess of $GABA_{B2}$ and, therefore, G protein signaling of other GPCRs may be influenced. High KCTD12 expression levels have been reported during development (Metz et al., 2011; Resendes et al., 2004) and may also occur in certain neurons (Metz et al., 2011) or under pathological conditions. Interestingly, KCTD12 has been linked to schizophrenia (Benes, 2010), bipolar disorder 1 (Lee et al., 2011), depression (Sibille et al., 2009; Surget et al., 2009), anxiety (Le-Niculescu et al., 2011), and gastrointestinal tumors (Hasegawa et al., 2013), which may not necessarily entail an exclusive action of KCTD12 at $GABA_B$ receptors.

Implications of G Protein Binding by the KCTDs for $GABA_B$ Receptor Signaling

KCTD12 displays effects on $GABA_B$ responses that may not directly relate to its role in Gβγ inhibition. Thus, all KCTDs accelerate the rise time (Schwenk et al., 2010) and reduce the latency (Figure S7) of baclofen-activated Kir3 currents in heterologous cells. Accordingly, their loss in *Kctd8/12/16* triple knockout mice leads to markedly slower rise times and increased latency of the K^+ currents in cultured hippocampal neurons (Figures 8C and 8D). The KCTD-dependent acceleration of the receptor response may result from the dual binding of the KCTDs to the receptor and the G protein. Pulling together receptor and G protein should overcome the slow diffusion-limited association between receptor and G protein and shift the rate-limiting step in G protein activation from receptor/G protein binding to receptor-driven GDP-GTP exchange, a faster process (Fowler et al., 2007; Ross, 2008). However, the KCTDs may also speed up G protein signaling at the receptor by directly accelerating GDP-GTP exchange, for example, by promoting the release of GDP from Gα·GDP. We recently reported that KCTD12 additionally promotes surface expression of $GABA_B$ receptors in neurons (Ivankova et al., 2013). The KCTDs therefore influence $GABA_B$ receptor signaling and thus physiological processes in several ways. First, KCTD12-induced fast activation kinetics may be important for a precise timing of pre- and postsynaptic $GABA_B$ receptor influences on synaptic transmission. Second, KCTD12-induced fast desensitization may serve to prevent excessive Kir3 channel activity, which can cause intracellular K^+ depletion and neuronal apoptosis (Yu et al., 1997) or generate seizures (Beenhakker and Huguenard, 2010). Third, the receptor specificity of KCTD12 may provide a means to avoid promiscuous and potentially adverse interference with the signaling of other GPCRs (Rives et al., 2009).

EXPERIMENTAL PROCEDURES

Molecular Biology and Cell Culture

The cDNAs encoding WT and mutant proteins used were all verified by sequencing and had the following GenBank accession numbers: Y10370 ($GABA_{B1b}$), AJ011318 ($GABA_{B2}$), AY615967 (KCTD8), AY267461 (KCTD12), and NM_026135 (KCTD16). The cDNAs encoding the Kir3.1/3.2 concatemer (Kaupmann et al., 1998) and the adenosine A1 receptor (Ferré et al., 2002) were reported earlier. Cell culturing and transfections were as described in Biermann et al. (2010), Ivankova et al. (2013), and Schwenk et al. (2010).

Biochemistry

Affinity purifications (APs), two-dimensional BN-PAGE/SDS-PAGE separations, and western blot analyses were carried out as described in Schwenk

et al. (2010, 2012). Protein samples for APs were obtained by solubilizing plasma membrane-enriched protein fractions from whole mouse brains with ComplexioLyte-47 (CL-47, Logopharm GmbH) at 1 mg protein/ml or by lysing cultured HEK293T cells or homogenized mouse brains with Nonidet P-40 buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris/HCl [pH 7.4]). AlF_4^- (60 μm $AlCl_3$, 10 mm NaF, 10 μm GDP, 10 mM $MgCl_2$) was freshly prepared as described in Kawano et al. (2007) and incubated in Nonidet P-40 buffer before lysis of HEK293T cells. Crude membrane preparations from *Xenopus* oocytes injected with the indicated cRNA were obtained as described previously in Schwenk et al. (2010).

Mass Spectrometry

Nano LC-MS/MS analyses were performed on an LTQ-FT Ultra mass spectrometer linked to an UltiMate 3000 HPLC as described (Schwenk et al., 2012). LC-MS/MS data were extracted using the extract_msn utility and searched against manually assembled databases derived from UniProt Knowledgebase release 2013/02 (*Mus musculus*, *Rattus norvegicus*, and *Homo sapiens*) using the Mascot search engine (version 2.3.01; Matrix Science) first with a peptide mass tolerance of 15 ppm. After extraction and mass shift calibration of precursor m/z signals using MaxQuant (Cox and Mann, 2008), tolerance was reduced to ±5 ppm for final searches.

Relative quantification of proteins was based on peptide peak volumes (PVs). PVs from individual peptide species were calculated from the respective LC-MS full-scan m/z signal intensities integrated over time and mass width either with MaxQuant (protein ratios in Figure 2) or msInspect (profiles in Figure 7). Alignment of m/z signals between different LC-MS/MS runs and assignment to the peptides identified by Mascot (retention time tolerance: 1 min, m/z difference threshold: ±2.5 ppm (MaxQuant), ±5 ppm (msInspect) was carried out by a home-written software tool and manually verified for proteins yielding less than six peptide PVs.

Protein abundance ratios (rPV; Figure 2) were determined by the TopCorr method as described in Bildl et al. (2012). Specificity thresholds of APs were determined from rPV histograms of all proteins detected in the respective AP/control. Proteins were considered specifically copurified when rPV (mouse WT versus KO)/threshold (versus KO) > 1. Unless indicated otherwise, only proteins with rPVs based on at least two protein-specific peptide PVs were quantified. Protein mass abundance profiles (Figure 7) were determined from BN-MS analysis as described (Schwenk et al., 2012).

BRET Measurements

BRET measurements were performed in CHO cells stably expressing $GABA_{B1}$ and $GABA_{B2}$ and transiently transfected with plasmids encoding Gαo-*R*luc, Venus-Gγ2, FLAG-Gβ2, and myc-KCTD12 or myc-KCTD16. BRET signals between Gαo-*R*luc and Venus-Gγ2 in the presence of 5 μM coelenterazine h (NanoLight Technologies) were measured on an Infinite F500 microplate reader (Tecan) after receptor activation with baclofen.

Electrophysiology

Experiments on *Xenopus* oocytes, CHO cells, and cultured hippocampal neurons were performed at room temperature as described in Schwenk et al. (2010).

Desensitization time constants were derived from double-exponential fits to the decay phase of Kir3.1/3.2 currents during baclofen application. Curve fitting and further data analyses were done with pClamp 10 (Molecular Devices) and IGOR Pro (version 6.32; Wavemetrics). Latency was determined in current responses filtered to 20 Hz as the time interval between the agonist solution reaching the cell surface and the inflection point indicating current onset (Doupnik et al., 2004). The inflection point was set at the last zero crossing of the first derivative of the current before the onset. Data are given as mean ± SD. Statistical significance was assessed using nonparametric t tests or ANOVA with the Dunnett's multiple comparison test. Additional information is provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.04.015.

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Supplemental Information

Auxiliary $GABA_B$ Receptor Subunits Uncouple G Protein βγ Subunits from Effector Channels to Induce Desensitization

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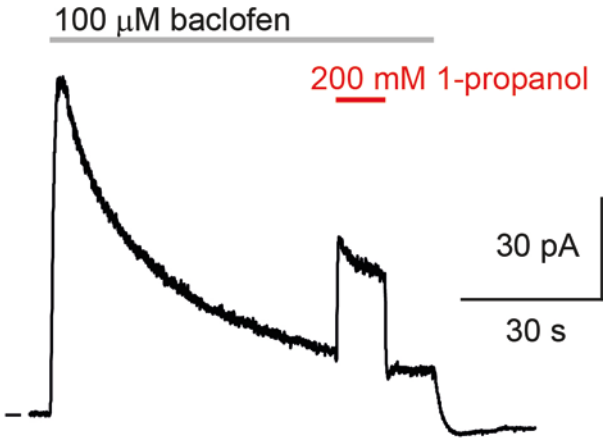
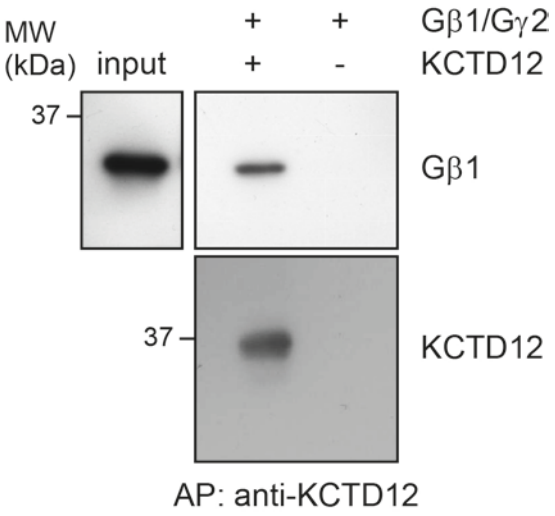
SUPPLEMENTAL FIGURES

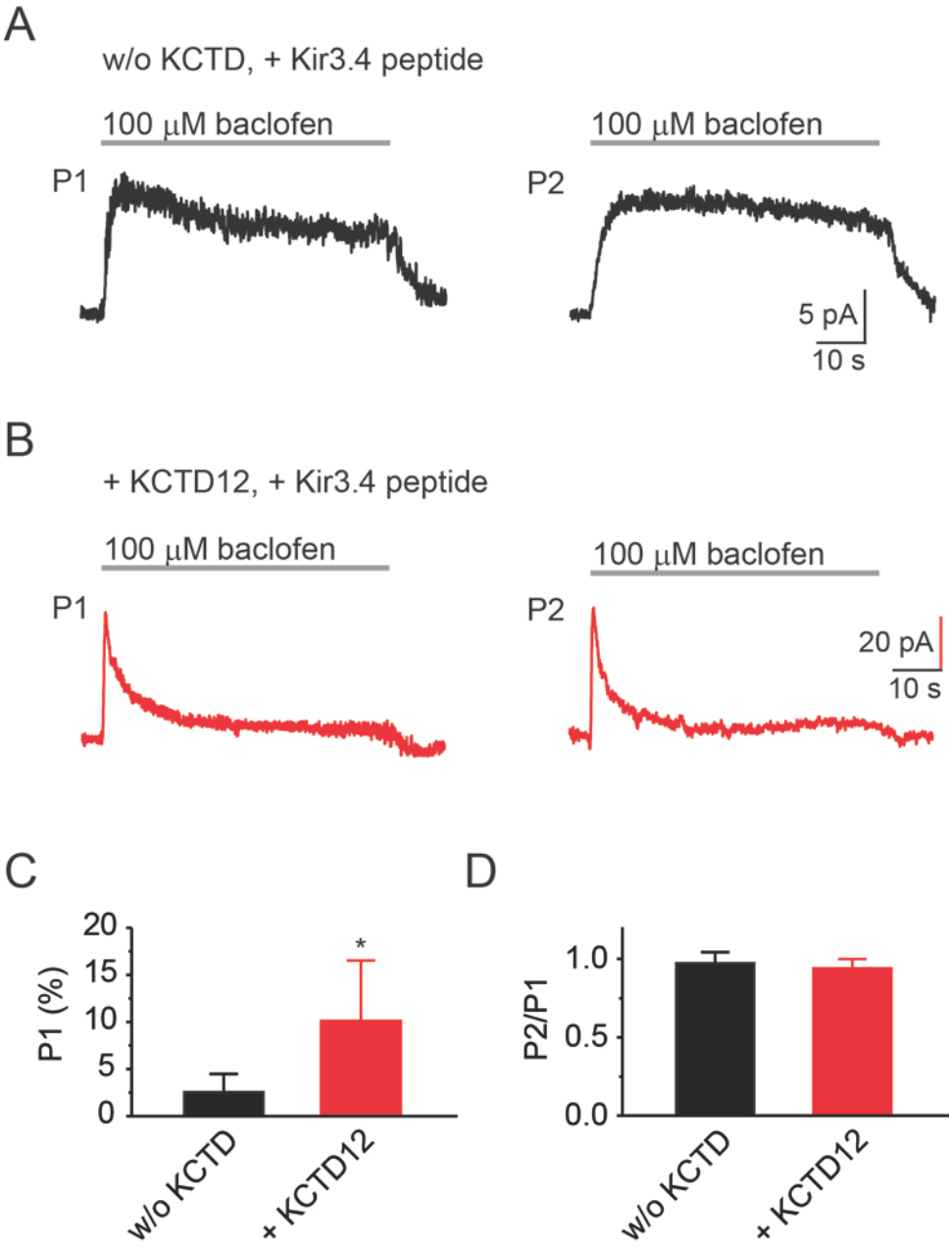
Supplemental Figure 1, related to Figure 1.



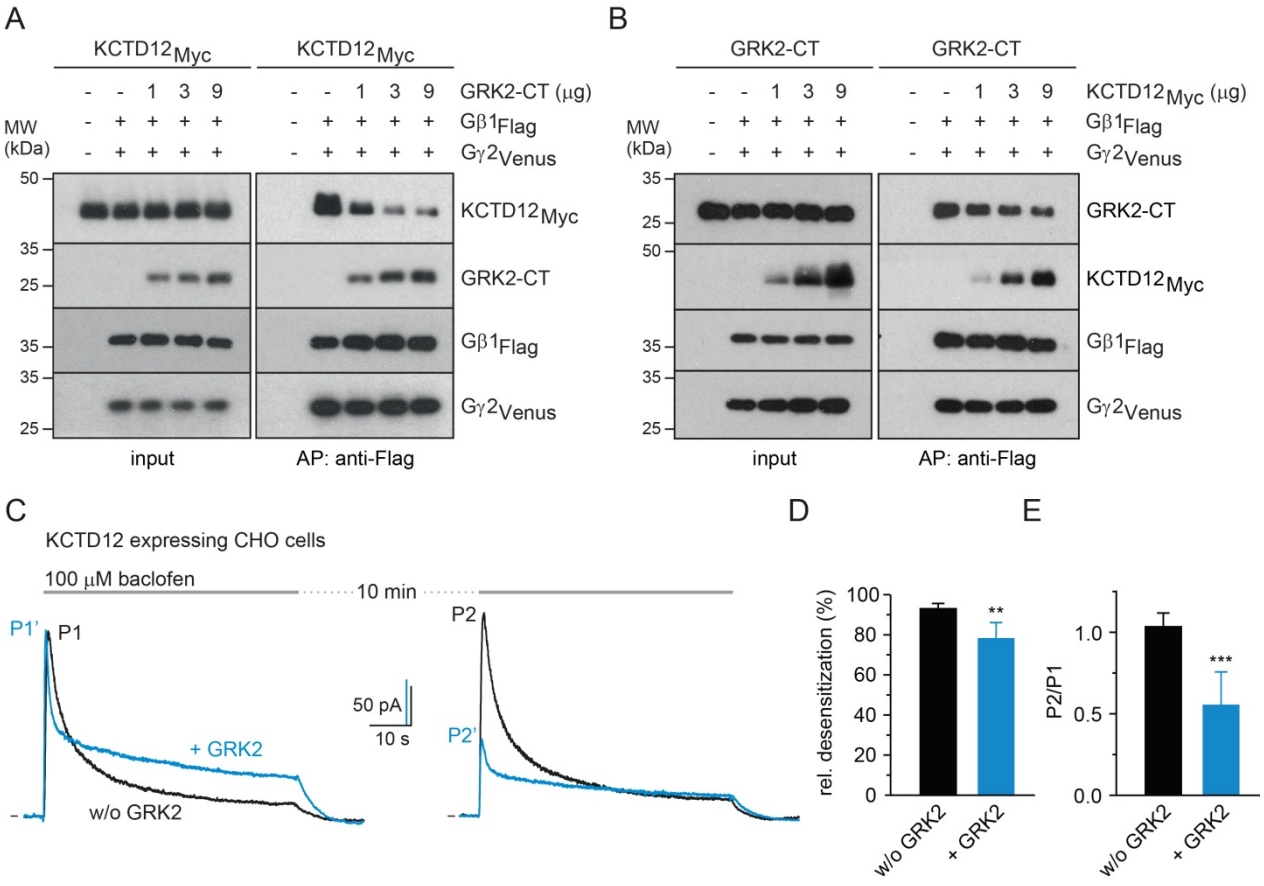
Supplemental Figure 2, related to Figure 2.



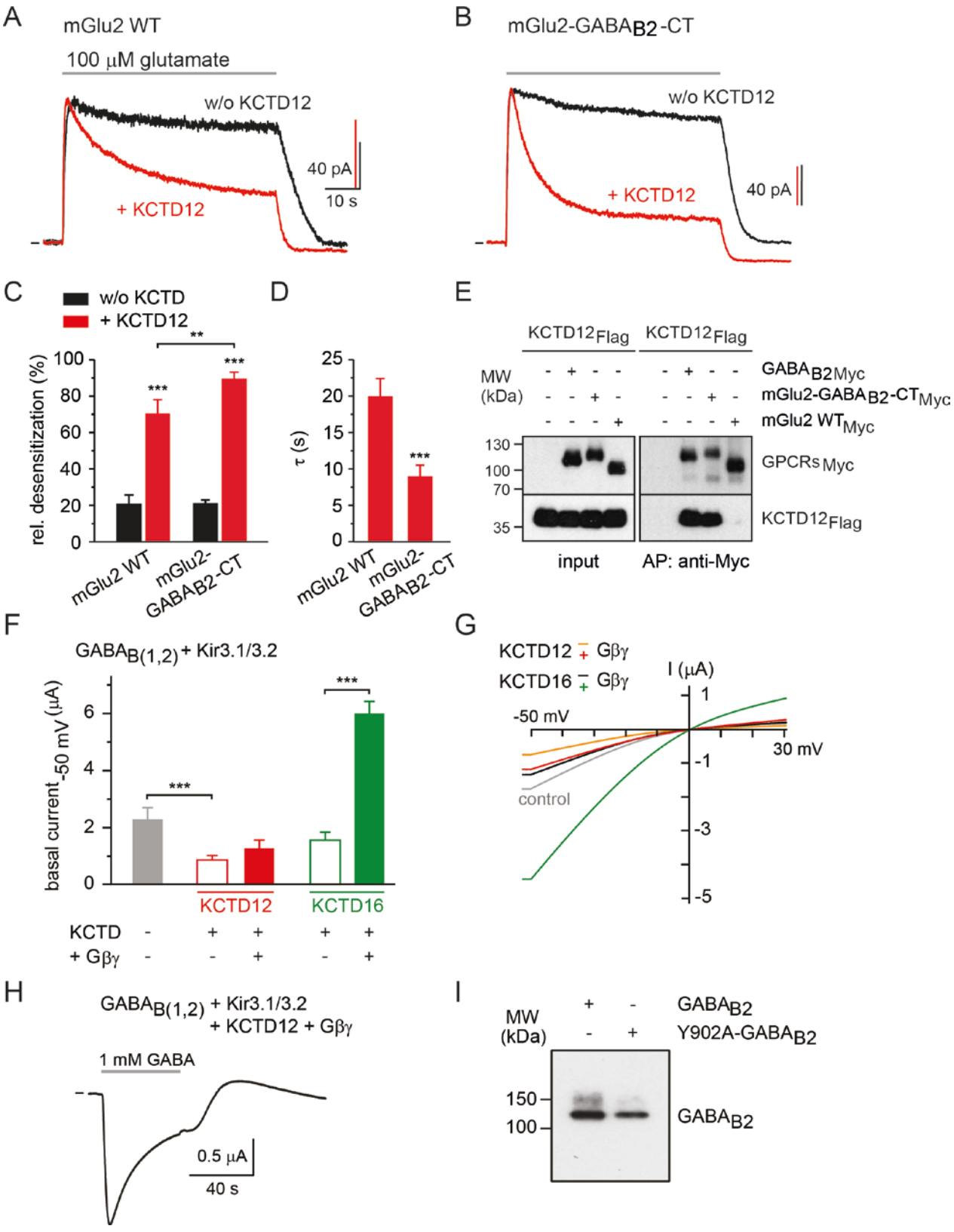
Supplemental Figure 3, related to Figure 4.



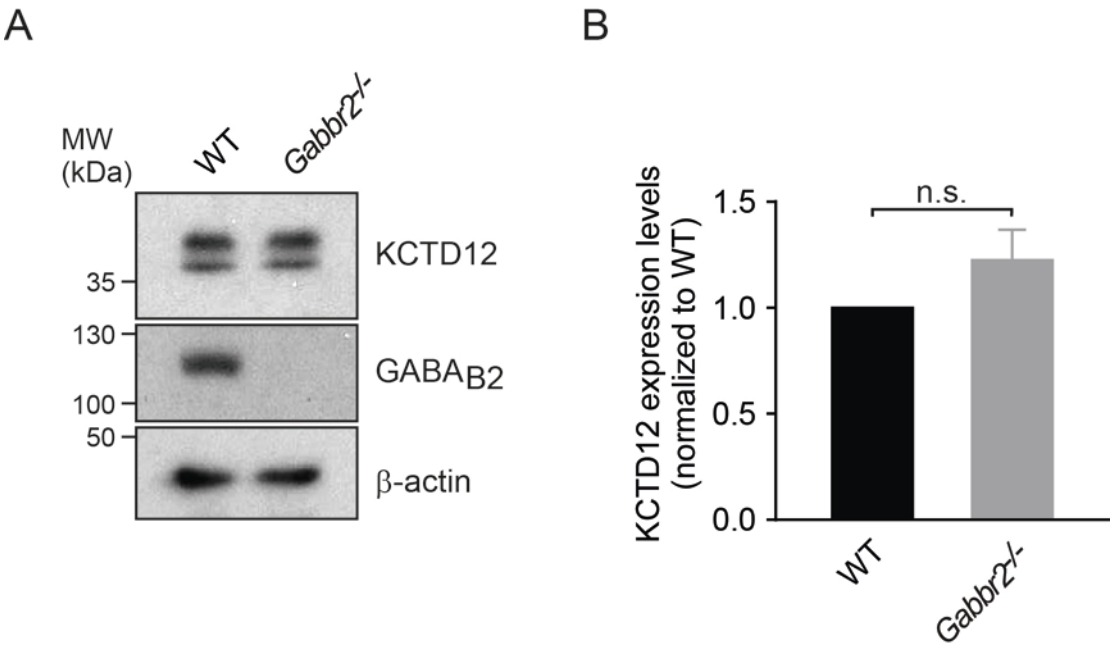
Supplemental Figure 4, related to Figure 5.



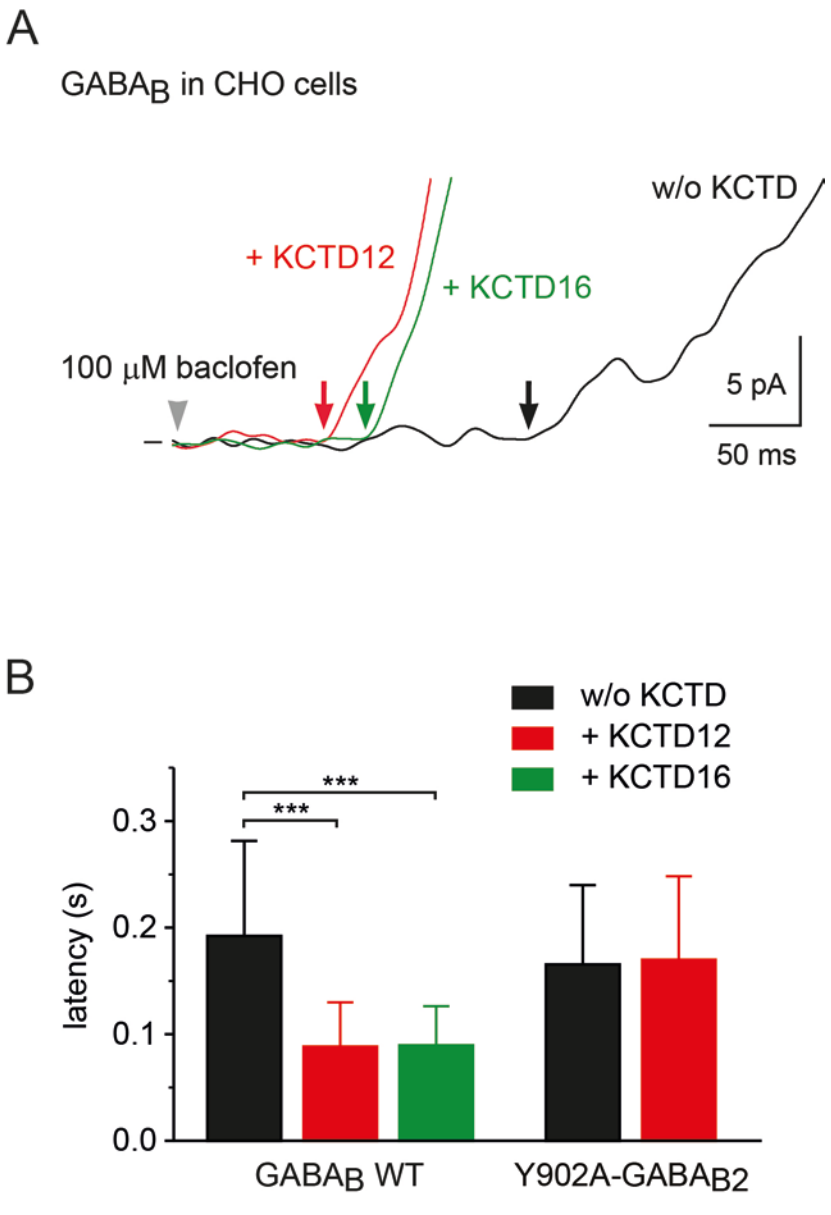
Supplemental Figure 5, related to Figure 6.



Supplemental Figure 6, related to Figure 7.



Supplemental Figure 7, related to Figure 8.



SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (Related to Figure 1).

Kir3.2 channels can be activated by 1-propanol during KCTD12-induced desensitization. Trace of Kir3 current recorded at -50 mV in CHO cells expressing $GABA_B$ receptors, Kir3.2 channels and KCTD12. Note that direct activation of Kir3 channels by 1-propanol is possible during KCTD12-induced desensitization of the current evoked by baclofen, showing that KCTD12 does not operate at the channel.

Figure S2 (Related to Figure 2).

Purified recombinant KCTD12 and Gβ1γ2 proteins directly interact with each other. Gβ1γ2 and KCTD12 proteins were expressed in baculovirus infected Sf21 insect cells and *E.coli,* respectively. The purified proteins were mixed in equimolar amounts, immunoprecipitated with KCTD12-specific antibodies and visualized on Western blots.

Figure S3 (Related to Figure 4).

KCTD12 does not promote unbinding of Kir3.4-derived peptide from Gβγ. (A, B) Traces represent Kir3 currents evoked by two consecutive baclofen applications delivered at a 5-minute interval to CHO cells expressing $GABA_B$ receptors in the absence (A) or presence of KCTD12 (B). Prior to the recordings the cells were perfused for 60 minutes with the intracellular solution supplemented with 40 μM Kir3.4 peptide. (C) Bar graph showing peak amplitudes (P1) of baclofen responses recorded after perfusion of cells with the Kir3.4 peptide relative to peaks of control baclofen-evoked currents recorded shortly after establishing of the whole-cell configuration. Note a lower sensitivity of the responses to the Kir3.4 peptide in the presence of KCTD12, consistent with competition between the peptide and KCTD12 for binding to Gβγ. Data

are mean ± SD of 7 (w/o KCTD) and 6 (+KCTD12) experiments. *, $p < 0.01$; Student's t-test. (D) Summary of the amplitude ratios of peak currents recorded during the second (P2) and first (P1) baclofen application. KCTD12 does not significantly alter the P2/P1 ratio indicating that it cannot rescue pre-inhibited Gβγ by reducing the affinity of Kir3.4-peptide at Gβγ. Data are mean ± SD of 5 (w/o KCTD, +KCTD12) experiments.

Figure S4 (Related to Figure 5).

The binding domains of KCTD12 and GRK2 on Gβγ overlap. (A, B) Flag-tagged Gβ1 ($Gβ1_{Flag}$) and Venus-tagged Gγ2 ($Gγ2_{Venus}$) were expressed in the presence of either (A) increasing amounts of GRK2 C-terminus (GRK2-CT, 1 - 9 µg of DNA) and equal amounts of Myc-tagged KCTD12 or (B) increasing amounts of $KCTD12_{Myc}$ (1 – 9 µg of DNA) and equal amounts of GRK2-CT. APs were analyzed by Western blot using antibodies directed at GRK2-CT, Myc, Flag, and GFP. MW, molecular weight; Venus, variant of GFP; CT, C-terminus. Note that GRK2 is more efficient in displacing KCTD12 from Gβγ than *vice versa*. (C) Kir3 currents evoked by two consecutive baclofen applications delivered at 10 minute interval to CHO cells expressing $GABA_B$ receptors and KCTD12 with (blue) or without (black) GRK2. Note that the combined presence of KCTD12 and GRK2 accelerates fast desensitization when compared to GRK2 alone (Figure 5). (D) Bar graph summarizing Kir3 current desensitization. Data are mean ± SD of 6 (w/o GRK2) and 5 (GRK2) experiments. ***, $p < 0.001$; **, $p < 0.01$; Student's t-test. (E) Bar graph summarizing the amplitude ratios of peak Kir3 currents recorded during the second (P2) and first (P1) baclofen application (panel (C)). Note that during the 10 minute period GRK2-induced desensitization does not recover in the presence of KCTD12. This shows that KCTD12 does not efficiently interfere with GRK2 mediated desensitization. Data are mean ± SD of 5 (w/o GRK2) and 9 (GRK2) recordings. ***, $p < 0.001$; Dunnett's multiple comparison test.

Figure S5 (Related to Figure 6)

Receptor-specific and receptor-unspecific KCTD12-induced desensitization. (A, B) Representative traces of Kir3 currents evoked by fast application of L-glutamate to CHO cells expressing WT metabotropic glutamate receptor 2 (mGlu2 WT) or mGlu2-$GABA_{B2}$-CT, in which the C-terminal 58 amino-acid residues of mGlu2 (accession number NM_001105711) were replaced with the C-terminal 197 residues of $GABA_{B2}$. Currents were recorded in the presence (+ KCTD12) and absence of KCTD12 (w/o KCTD12). (C) Bar graph summarizing Kir3 current desensitization. Note that binding of KCTD12 to mGlu2-$GABA_{B2}$-CT increases the extent of desensitizsation. Data are mean ± SD of 5 experiments for each treatment. ***, $p < 0.001$; *, $p < 0.01$; Student's t-test. (D) Bar graph shows mean amplitude-weighted time constants obtained from a fit of a double exponential function to KCTD12-induced desensitization decay. Note that binding of KCTD12 to mGlu2-$GABA_{B2}$-CT accelerates desensitization of the current response. Data are mean ± SD of 5 experiments for each treatment. ***, $p < 0.001$; Student's t-test. (E) KCTD12 co-immunoprecipitates with mGlu2-$GABA_{B2}$-CT_{Myc}. Flag-tagged KCTD12 was expressed together with either Myc-tagged $GABA_{B2Myc}$, mGlu2 WT_{Myc} or mGlu2-$GABA_{B2}$-CT_{Myc}. APs were performed with *anti-Myc* antibodies and analyzed on Western blot with antibodies against Myc and Flag. *MW,* molecular weight. (F) Effect of KCTD12 on basal Kir3 currents expressed in *Xenopus* oocytes. Amplitudes of basal Kir3 currents recorded from whole oocytes injected with cRNA encoding $GABA_B$ receptors and Kir3.1/3.2 channels in the absence (control) or presence of KCTD12, KCTD16 and Gβγ. Bars are mean ± SEM of 6 oocytes for each condition; ***, $p < 0.001$; Mann-Whitney U-test. Note that KCTD12 but not KCTD16 significantly reduced basal currents mediated by both endogenous and overexpressed exogenous Gβγ. (G) Representative recordings of Kir3 currents elicited by voltage ramps (from -50mV to 30mV). Amplitudes recorded at -50 mV are summarized in (F). (H) Effect of overexpressed KCTD12 on receptor-activated Kir3 currents in *Xenopus* oocytes. Representative $GABA_B$-activated Kir3 currents recorded at -50 mV from *Xenopus* oocytes injected with cRNA encoding $GABA_B$

receptors, Kir3.1/3.2, KCTD12 and Gβγ. Note robust KCTD12-mediated desensitization of the current response in the presence of overexpressed Gβγ. (I) Membrane fractions of *Xenopus* oocytes injected with the cRNAs encoding WT or Y902A-$GABA_{B2}$ analyzed by Western blot with an *anti-$GABA_{B2}$* antibody.

Figure S6 (Related to Figure 7).

Total KCTD12 protein levels are not significantly altered in *Gabbr2*$^{-/-}$ compared to WT mouse brains. (A) Western blots of total protein extracts from the brains of a *Gabbr2*$^{-/-}$ and WT littermate mouse. β-actin served as a loading control. (B) Bar graphs summarizing KCTD12 expression levels in *Gabbr2*$^{-/-}$ and WT mice, normalized to WT expression levels. Data are mean ± SD of 3 independent experiments ($p = 0.25$, Wilcoxon signed-rank test).

Figure S7 (Related to Figure 8)

KCTD12 and KCTD16 reduce the latency of baclofen-activated Kir3 currents in transfected CHO cells. (A) Representative traces of baclofen-evoked currents recorded from CHO cells expressing $GABA_B$ receptors and Kir3 channels with or without (w/o) KCTD proteins. The latency period between start of the agonist application (arrow head) and current onset (arrows) is reduced in the presence of KCTD12 and 16. Current and time scale as indicated. (B) Bar graph summarizing the latency of baclofen-evoked Kir3 currents observed with WT and mutant Y902A-$GABA_{B2}$ receptors. The latency is only reduced by KCTD12 and KCTD16 in the presence of WT receptors. Data are mean ± SD ***, $p < 0.001$; Student's t-test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Biochemistry

Affinity purifications and Western blot analyses were carried out as described (Ivankova et al., 2013; Schwenk et al., 2012). Briefly, plasma membrane-enriched protein fractions were prepared from whole brains of adult WT mice, as well as from *Gabbr2*$^{-/-}$ and *Kctd12*$^{-/-}$ mice and solubilized for 30 minutes at 4°C with ComplexioLyte47 (CL-47, Logopharm GmbH, at 1 mg protein/ml). After clearing by ultracentrifugation (10 min at 150.000 x g), 1.5 ml of solubilizate was incubated with 15 µg of immobilized *anti-GABA*$_{B2}$ (Schwenk et al., 2010) or *anti-KCTD12* antibodies (Metz et al., 2011) for 2 h on ice. After two brief washes with CL-47, bound proteins were eluted in Laemmli buffer. The efficiency of solubilization and purification was controlled by Western blot analysis of SDS-PAGE resolved aliquots taken along this procedure. Two-dimensional BN-PAGE/SDS-PAGE separations were carried out as described (Schwenk et al., 2012) on linear 3-15% polyacrylamide gradient gels. Excised BN-PAGE lanes were incubated for 20 min in Laemmli buffer and placed on top of 10% SDS-PAGE gels. After electro-blotting on PVDF membranes the blot was cut horizontally into different molecular weight ranges and stained with the indicated antibodies.

For APs from cultured cells (Figures 2, 3, S4 and S5), HEK293T cells were harvested, washed in PBS, and subsequently lysed in a Nonidet P-40 buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris/HCl, pH 7.4) supplemented with complete EDTA-free protease inhibitor mixture (Roche). After rotation for 10 min at 4 °C, lysates were cleared by centrifugation at 16,000 × g for 10 min at 4 °C. The lysates were then used for Western blot analysis or precleared for 1 h using 30 µl dry volume of a 1:1 mixture of protein-A and protein-G agarose (GE Healthcare) for APs. Precleared lysates were immunoprecipitated with mouse *anti-Flag* (F1804, Sigma) or mouse *anti-Myc* (Figure S5, sc-40, Santa Cruz) antibodies and 10 µl of the 1:1 protein-A and protein-G agarose mixture, after 3 h of incubation at 4 °C. Lysates and

immunoprecipitates were resolved using SDS-PAGE, and probed with the primary antibodies rabbit *anti-Myc* (F1804, Sigma, 1:1000), mouse *anti-Flag* (F7425, Sigma, 1:1000), mouse *anti-Renilla Luciferase* (MAB4410, Millipore, 1:2000), rabbit *anti-GFP* (A11122, Invitrogen, 1:1000) or rabbit *anti-GRK2-CT* (ab32558, Abcam) and peroxidase-coupled secondary antibodies (NA931V and NA9340V, Amersham Biosciences, 1:10000). The antibody incubation was in 5% nonfat dry milk in PBS containing 0.1% Tween-20. The chemiluminescence detection kit (Pierce) was used to identify antibody-labeled proteins.

For the AlF_4^- experiment, AlF_4^- (60 µm $AlCl_3$ (449598, Sigma), 10 mm NaF (215309, Sigma), 10 µm GDP (G7127, Sigma) and 10 mM $MgCl_2$ (63064, Fluka)) was freshly prepared from powder as described (Kawano et al., 2007) and incubated in Nonidet P-40 lysis buffer before lysis of HEK293T cells as described above.

For determination of total KCTD12 protein levels (Figure S6) mouse brains were washed in ice-cold PBS buffer and mouse brain tissue was homogenized in Nonidet P-40 lysis buffer using a glass-teflon homogenizer with 20 passes on ice. After rotation for 10 min at 4 °C, homogenates were clarified by centrifugation at 16,000 × g for 10 min at 4 °C. The supernatant was retained and used for protein determination by Bradford method or Western blot analysis. Lysates were resolved using SDS-PAGE, and probed with the primary antibodies rabbit *anti-KCTD12* (Schwenk et al., 2010), rabbit *anti-*$GABA_{B2}$ (AGB-002, Alomone, 1:1000) or rabbit *anti-β-actin* (4970, Cell Signaling Technology, 1:2000). Band intensity was quantified by ImageJ software (National Institute of Health, Bethesda, MD, USA) and depicted in graphical form.

Histidine tagged human Gγ2 and Gγ1 were subcloned into multigene baculovirus transfer vectors (Fitzgerald et al., 2006) and subsequently integrated into the bacmid genome. Virus mediated protein expression was conducted by infecting Sf21 insect cells. Cells were harvested and lysed with glas potters in homogenization buffer consisting of 10 mM Tris/HCl pH 7,4, 320 mM Sucrose, 1,5 mM MgCl, 1 mM EGTA, 1 mM TCEP with freshly added protease inhibitors.

Membrane fractions were isolated by ultracentrifugation (100.000 x g, 30 minutes). Proteins were solubilized as described in (Kozasa and Gilman, 1995), purified by Ni-Sepharose (GE Healthcare) and size-exclusion chromatography (Superdex 200, GE Healthcare) and thereafter dialysed against binding buffer (20 mM Tris/HCl pH 7,4, 150 mM NaCl, 0,1 mM TCEP, 0,15% Zwittergent 3-14). His-tagged KCTD12 was expressed in *E.coli* BL21(DE3) pLysS. Cells were lysed by sonication and soluble KCTD12 protein was purified sequentially by IMAC and SEC chromatography and dialysed against binding buffer. Equimolar amounts of G proteins and KCTD12 were mixed in binding buffer and after 30 min immunoprecipitated with 2 µg immobilized KCTD12-specific antibodies. Samples were analysed by Western blot.

Crude membrane preparations from *Xenopus* oocytes injected with the indicated cRNA were obtained as described previously (Schwenk et al., 2010). Equal amounts of membrane proteins were separated on a 10% SDS-PAGE and analyzed by Western blot.

Mass Spectrometry

Analysis by nano LC-MS/MS was performed as described (Schwenk et al., 2012). Specifically, peptide mixtures obtained from tryptic in-gel digestion of protein samples (dissolved in 0.5% (v/v) trifluoroacetic acid) were resolved by reverse phase chromatography on an UltiMate 3000 HPLC and directly electrosprayed into an LTQ FT Ultra mass spectrometer. Precursor signals (full scans) used for quantification were acquired with a target value of 1,000,000 (maximum injection time 1 s, spray voltage 2.3 kV; capillary temperature 125°C) and a nominal resolution of 100,000 (FWHM) at m/z 400 (scan range 370 to 1700 m/z). Up to five data-dependent CID fragment spectra per scan cycle were acquired in the ion trap with a target value of 10,000 with dynamic exclusion, preview mode for FTMS master scans, charge state screening, monoisotopic precursor selection and (+1) charge state rejection enabled. Total MS acquisition times were 105 min (75 min rising acetonitrile concentration, 30 s dynamic MS/MS exclusion). LC-MS/MS

data were extracted using the extract_msn utility (grouping tolerance 0.05) and searched against (manually assembled databases) derived from UniProt Knowledgebase release 2013/02 (*Mus musculus*, *Rattus norvegicus* and *Homo sapiens*) using the Mascot search engine (version 2.3.01; Matrix Science) first with a peptide mass tolerance of 15 ppm. After extraction and mass shift calibration of precursor m/z signals using MaxQuant (Cox and Mann, 2008), tolerance was reduced to ± 5 ppm for final searches. One missed trypsin cleavage and common variable modifications were accepted for peptide identification. Proteins identified by only one specific MS/MS spectrum or representing exogenous contaminations such as keratins or immunoglobulins were eliminated.

Relative quantification of proteins was based on peptide peak volumes (PVs). PVs from individual peptide species were calculated from the respective LC-MS full scan m/z signal intensities integrated over time and mass width either with MaxQuant (protein ratios in Figure 2) or msInspect (http://proteomics.fhcrc.org/CPL/amt/), Computational Proteomics Laboratory, Fred Hutchinson Cancer Research Center, Seattle, WA, USA; protein profiles in Figure 7). Alignment of m/z signals between different LC-MS/MS runs and assignment to the peptides identified by Mascot (retention time tolerance: 1 min, m/z difference threshold: ± 2.5 ppm (MaxQuant), ± 5 ppm (msInspect) was carried out by a home-written software tool and manually verified for proteins yielding less than 6 peptide PVs. Protein abundance ratios (rPV) in AP versus control (Figure 2) were determined by the TopCorr method as described (Bildl et al., 2012). Specificity thresholds of APs were determined from rPV histograms of all proteins found in the respective AP / control. Proteins were considered specifically co-purified when rPV (mouse WT versus KO) / threshold (vs KO) > 1 (Figure 2). Unless indicated otherwise, only proteins with rPVs based on at least two protein-specific peptide PVs were quantified. Protein mass abundance profiles (i.e. protein abundance ratios of each gel slice sample versus maximum (without calibration of molecular abundances, Figure 7) were determined from BN-MS analysis as described (Schwenk

et al., 2012). Gaussian least square fits to protein profiles were carried out with IGOR Pro (version 6.32; Wavemetrics USA).

BRET Measurements

CHO cells expressing $GABA_{B1}$ and $GABA_{B2}$ were transiently transfected with plasmids encoding Gαo-*R*luc, Venus-Gγ2, FLAG-Gβ2, and myc-KCTD12 or myc-KCTD16 and were seeded into 96-well microplates (Greiner Bio-One). 24 h after transfection, cells were washed with PBS and the measurement was initiated on an Infinite® F500 microplate reader (Tecan) after injection of 5 µM Coelenterazine h (NanoLight Technologies). 100 µM baclofen was injected after 2 min of reading. Luminescence and fluorescence signals were detected sequentially with an integration time of 200 ms. The expression of $GABA_B$ receptors at the cell surface was controlled with an ELISA (Ivankova et al., 2013) and did not differ between experimental conditions. The BRET ratio was calculated as the ratio of light emitted by Venus-Gγ2 (530-570 nm) over light emitted by Gαo-*R*luc (370-470 nm) and corrected by subtracting ratios obtained with the *R*luc fusion protein alone. The results were expressed in mBRET units. The curves were fitted using GraphPad Prism 5.0 (“Plateau followed by one-phase decay”).

Electrophysiology

Experiments on *Xenopus* oocytes, CHO cells and cultured hippocampal neurons were performed at room temperature as previously described (Schwenk et al., 2010). In two-electrode voltage-clamp experiments on oocytes, the extracellular solution was composed as follows (mM): 100 KCl, 17.5 NaCl, 10 HEPES, and 1.8 CaCl2 (pH 7.3); solution exchange occurred within ~1 s. For experiments with CHO cells and hippocampal neurons cells were continuously superfused with an extracellular solution composed of (in mM): 145 NaCl, 2.5 KCl, 1 MgCl2, 2

CaCl2, 10 HEPES, 25 Glucose; pH 7.3, 323 mOsm. Patch pipettes had resistances between 3-4 MΩ when filled with intracellular solution composed of (in mM) 107.5 potassium gluconate, 32.5 KCl, 10 HEPES, 5 EGTA, 4 MgATP, 0.6 NaGTP (LiGTPγS), 10 Tris phosphocreatine; pH 7.2, 297 mOsm. Series resistance (< 5 MΩ) was compensated by 80%. Kir3 responses were evoked by fast application of baclofen (Schwenk et al., 2010) and recorded with an Axopatch 200B patch-clamp amplifier (Molecular Devices, USA); filtering and sampling frequencies were set to 1 kHz and 5 kHz, respectively. Solution exchange occurred within ~12 ms. To establish the *Kctd8/12/16*$^{-/-}$ line single-knockout mice were crossed. Homozygous *Kctd8/12/16*$^{-/-}$ mice from this line were compared with WT mice derived from heterozygous single knockout mice.

Desensitization time constants were derived from double-exponential fits to the decay phase of Kir3.1/3.2 currents during baclofen application. Curve fitting and further data analyses were done with pClamp 10 (Molecular Devices, USA) and IGOR Pro (version 6.32; Wavemetrics USA). Data are given as mean ± SD. Statistical significance was assessed using non-parametric t-tests or ANOVA with the Dunnett's multiple comparison test.

The Kir3.4-peptide (SMRDEKLCLMFRVGDLR, Biomatik, USA) was dissolved in intracellular solution at a final concentration of 40 µM. In experiments with gallein (Tocris Bioscience, UK) or selenocystamine dihydrochloride (Sigma-Aldrich, USA), CHO cells were preincubated for 30 to 60 minutes at room temperature in extracellular solution containing the drug at a concentration of 20 µM. For patch-clamp recording, the drugs were dissolved in intracellular solution at a concentration of 20 µM.

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2.2 $GABA_B$ receptor phosphorylation regulates KCTD12-induced K^+ current desensitization

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Personal contribution

Design of experiments

Co-immunoprecipitations and Western blot analysis

BRET measurements

Preparation of primary neuronal cultures

Transfection of cells for electrophysiology

Data analysis

Design of figures

Preparation of the manuscript

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$GABA_B$ receptor phosphorylation regulates KCTD12-induced K^+ current desensitization

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ABSTRACT

$GABA_B$ receptors assemble from $GABA_{B1}$ and $GABA_{B2}$ subunits. $GABA_{B2}$ additionally associates with auxiliary KCTD subunits (named after their K^+ channel tetramerization-domain). $GABA_B$ receptors couple to heterotrimeric G-proteins and activate inwardly-rectifying K^+ channels through the βγ subunits released from the G-protein. Receptor-activated K^+ currents desensitize in the sustained presence of agonist to avoid excessive effects on neuronal activity. Desensitization of K^+ currents integrates distinct mechanistic underpinnings. $GABA_B$ receptor activity reduces protein kinase-A activity, which reduces phosphorylation of serine-892 in $GABA_{B2}$ and promotes receptor degradation. This form of desensitization operates on the time scale of several minutes to hours. A faster form of desensitization is induced by the auxiliary subunit KCTD12, which interferes with channel activation by binding to the G-protein βγ subunits. Here we show that the two mechanisms of desensitization influence each other. Serine-892 phosphorylation in heterologous cells rearranges KCTD12 at the receptor and slows KCTD12-induced desensitization. Likewise, protein kinase-A activation in hippocampal neurons slows fast desensitization of $GABA_B$ receptor-activated K^+ currents while protein kinase-A inhibition accelerates fast desensitization. Protein kinase-A fails to regulate fast desensitization in KCTD12 knock-out mice or knock-in mice with a serine-892 to alanine mutation, thus demonstrating that serine-892 phosphorylation regulates KCTD12-induced desensitization in vivo. Fast current desensitization is accelerated in hippocampal neurons carrying the serine-892 to alanine mutation, showing that tonic serine-892 phosphorylation normally limits KCTD12-induced desensitization. Tonic serine-892 phosphorylation is in turn promoted by assembly of receptors with KCTD12. This cross-regulation of serine-892 phosphorylation and KCTD12 activity sharpens the response during repeated receptor activation.

1. Introduction

$GABA_B$ receptors are the G-protein coupled receptors (GPCRs) for GABA, the main inhibitory neurotransmitter in the central nervous system [1]. $GABA_B$ receptors are obligate heteromers composed of $GABA_{B1}$ (GB1) and $GABA_{B2}$ (GB2) subunits. Two GB1 isoforms exist, GB1a and GB1b, which regulate axonal versus dendritic distribution of the receptors [2]. In the heteromer GB1 binds GABA while GB2 facilitates surface trafficking and binds the G-protein [3–6]. $GABA_B$ receptors are expressed by most neurons in the brain and regulate neuronal excitability by modulating the activity of G protein-gated inwardly rectifying K^+ channels (GIRK or Kir3 channels), voltage-gated Ca^{2+} channels and adenylyl cyclase [1,7,8]. To avoid prolonged effects on neuronal activity in the continuous presence of GABA, $GABA_B$ receptors exhibit a time-dependent decrease in receptor response, a phenomenon referred to as homologous desensitization [9–12]. $GABA_B$ receptor activity induces homologous desensitization through the inhibition of adenylate cyclase, which in turn decreases the cAMP-dependent activity of protein kinase-A (PKA). Since PKA phosphorylation of serine-892 (S892) in the C-terminal domain of GB2 increases receptor stability at the plasma membrane, reduced PKA

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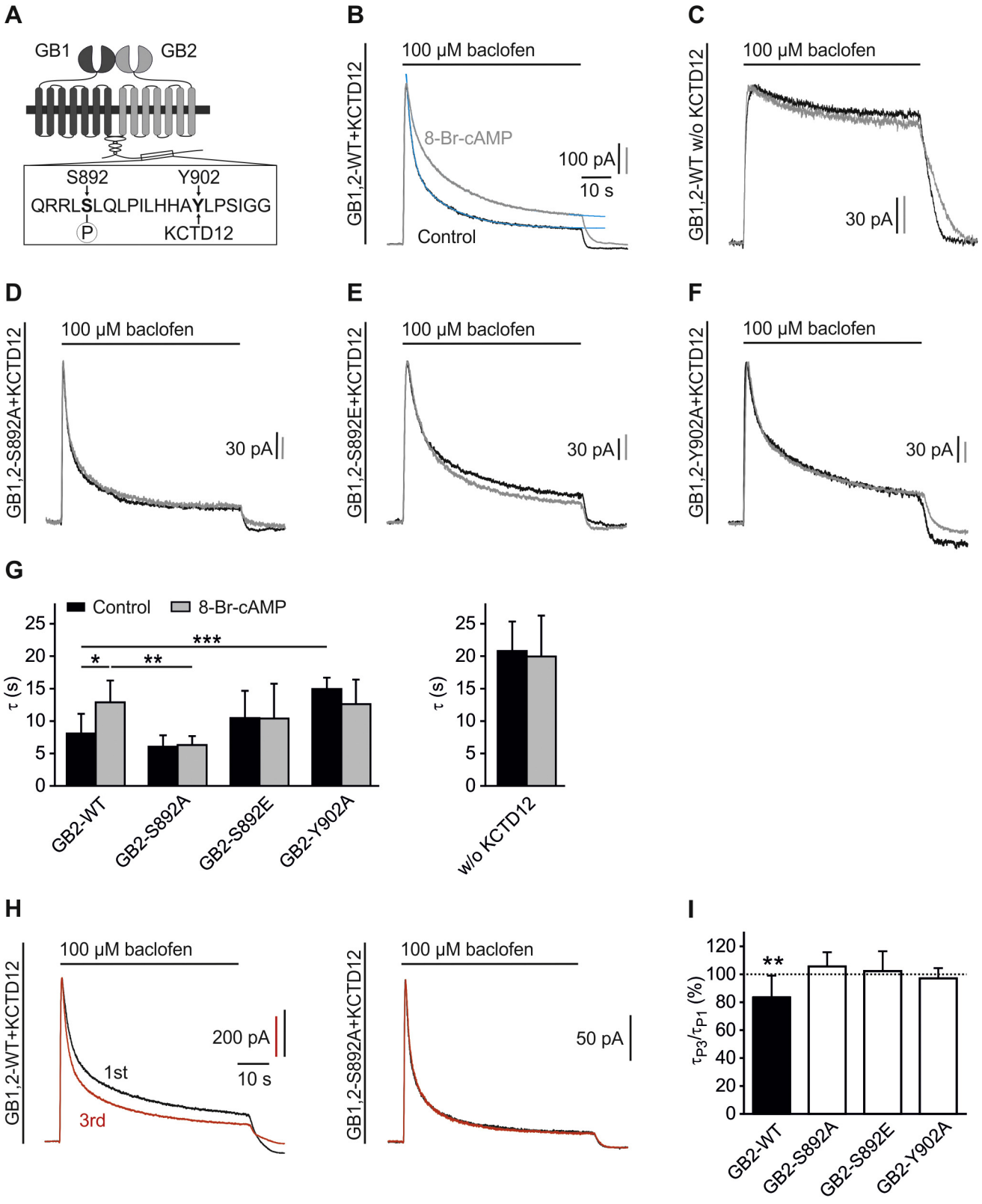

Fig. 1. S892 phosphorylation slows KCTD12-induced desensitization of baclofen-activated K^+ currents. (A) Scheme highlighting the close proximity of the PKA phosphorylation-site S892 and the KCTD12 binding-site Y902 in the C-terminal domain of GB2. (B) Representative traces of GIRK currents activated by baclofen (100 µM) and recorded at −50 mV from CHO-K1 cells expressing GB2-WT, GB1b, GIRK1/2 and KCTD12. Cells were pre-incubated without (Control, black trace) or with 8-Br-cAMP (1 mM, 30 min; grey trace). The desensitization time constants τ_1 and τ_2 were derived from double-exponential fits (blue trace) to the decay phase of K^+ currents during baclofen application. (C) Representative traces as in (B) from CHO-K1 cells lacking KCTD12. (D–F) Representative traces as in (B) from CHO-K1 cells expressing the GB2 mutants S892A (D), S892E (E) or Y902A (F). (G) Bar graph summarizing the time constant τ (amplitude-weighted mean time constant calculated from τ_1 and τ_2) of baclofen-induced K^+ current desensitization. Data are means ± SD, n = 5–16. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Sidak's multiple comparison test. (H) Repeated activation of GIRK currents in CHO-K1 cells expressing WT $GABA_B$ receptors (GB1,2-WT) together with KCTD12 results in a sharpening of the current response (left; 1st response black, 3rd response red). No sharpening of the current response upon repeated activation is observed with mutated $GABA_B$ receptors (GB1,2-S892A) that cannot be phosphorylated at S892 (right). Baclofen applications were

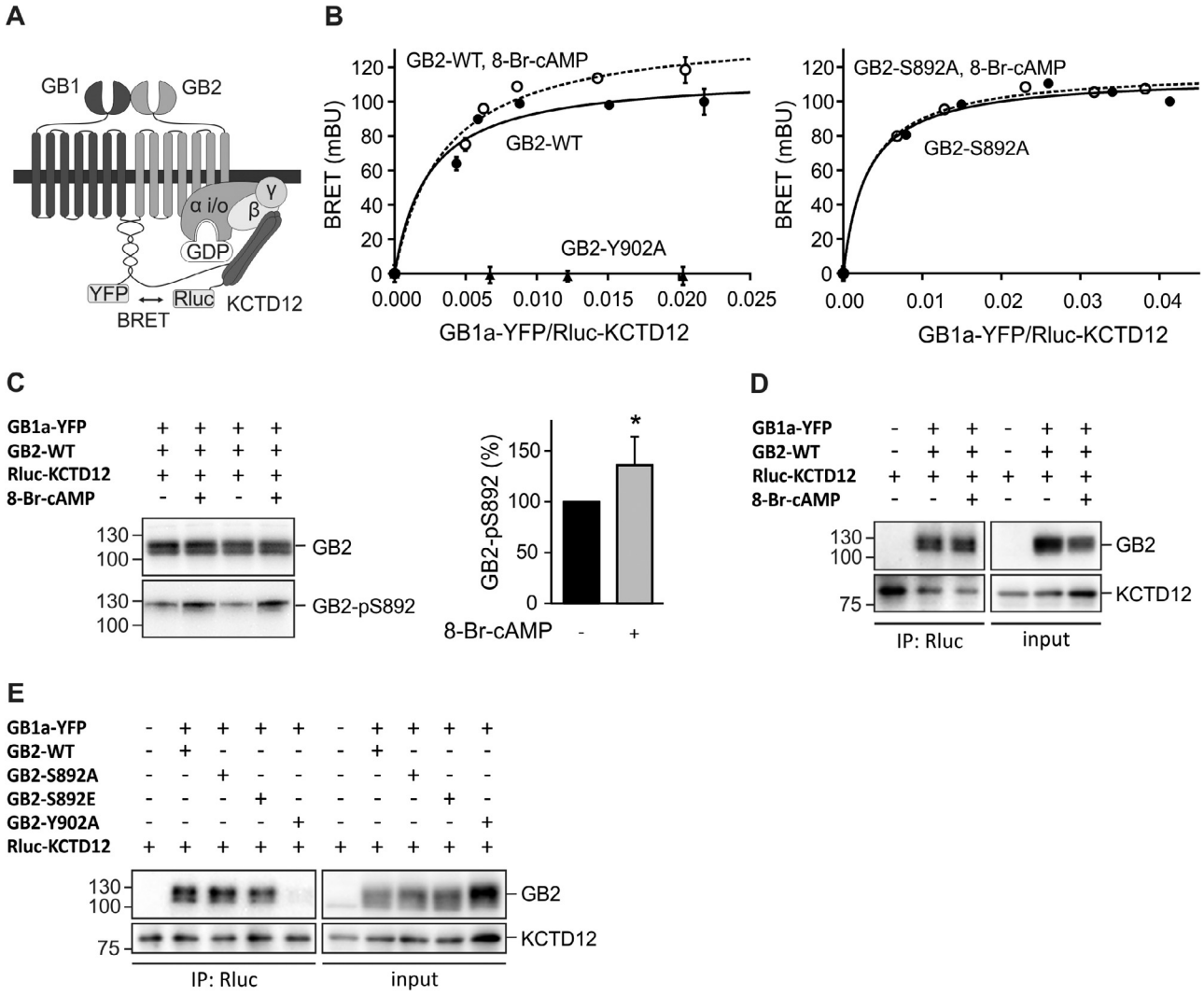

Fig. 2. S892 phosphorylation rearranges the receptor/KCTD12 complex. (A) Scheme of the BRET Rluc-KCTD12 donor and GB1a-YFP acceptor pair. (B) BRET donor saturation curves from HEK293T cells expressing fixed amounts of GB2-WT, GB2-Y902A (both left) or GB2-S892A (right) together with Rluc-KCTD12 and increasing amounts of GB1a-YFP. Cells were pre-incubated with 8-Br-cAMP (1 mM, 30 min; open circles) to activate PKA. BRET is expressed as milli BRET units (mBU) determined as net BRET × 1000. Data points are means ± SD of technical quadruplicates of a representative experiment, $n = 4–7$. (C) Western blot of lysates of HEK293T cells expressing GB1a-YFP, GB2-WT and Rluc-KCTD12 in the presence and absence of 8-Br-cAMP (1 mM, 30 min pre-incubation). Activation of PKA does not affect the total amount of GB2 but increases phosphorylation at S892 (GB2-pS892). GB2 runs as two bands on SDS–PAGE, likely due to differences in posttranslational modification. The higher molecular weight band appears to be more heavily phosphorylated at S892. Blots are representative of six independent experiments. Bar graph summarizing the amount of S892 phosphorylation normalized to the amount of GB2 on the same blot (GB2-pS892 in %). Data are means ± SD, $n = 6$. *, $p < 0.05$; t test. (D) Co-immunoprecipitation of $GABA_B$ receptors with KCTD12 from HEK293T cells expressing GB1a-YFP, GB2-WT and Rluc-KCTD12 in the presence and absence of 8-Br-cAMP (1 mM, 30 min pre-incubation). Equivalent amounts of $GABA_B$ receptors were co-precipitated. Blots are representative of five independent experiments. (E) Co-immunoprecipitation of $GABA_B$ receptors with KCTD12 from HEK293T cells expressing GB1a-YFP, GB2 (WT, S892A, S892E or Y902A) and Rluc-KCTD12. Y902A does not bind KCTD12 and was used as a negative control. Blots are representative of three independent experiments.

activity facilitates receptor degradation and consequently desensitization of the receptor response [13,14]. This phosphorylation-dependent mechanism regulates desensitization on the time scale of several minutes to hours. In addition, faster mechanisms induce homologous desensitization of the receptor response within seconds of agonist application. These mechanisms operate at the G-protein rather than at the receptor. The "Regulator of G-protein Signaling" (RGS) protein 4 induces desensitization by accelerating the rate of GTP hydrolysis at the Gα subunit of the activated G-protein [15–17]. Fast desensitization is additionally induced by the KCTD proteins 12 and 12b [18,19]. The KCTDs are auxiliary receptor subunits that constitutively associate with the C-terminal domain of GB2, in which mutation of Y902 to alanine (Y902A) completely abolishes association [18,20,21]. KCTD12 induces $GABA_B$ receptor-activated K^+ current desensitization by binding to the βγ subunits of the activated G-protein, which interferes with activation of the effector K^+ channel [19]. Of note, this post-receptor mechanism of desensitization is rendered receptor-specific through the exclusive association of native KCTD12 protein with $GABA_B$ receptors [19].

Since KCTD12 binds to GB2 in proximity of the PKA phosphorylation-site S892 (Fig. 1A), phosphorylation of S892

for 60-s in intervals of 5 min. (I) Bar graph summarizing experiments as shown in (H). In experiments with GB2-WT, the τ of the 3rd response (τ_{P3}) was significantly reduced compared to the τ of the 1st response (τ_{P1}). No change in the desensitization of the current response upon repeated activation is seen (i) when S892 cannot be phosphorylated (S892A), (ii) with the phospho-mimetic S892E and (iii) when KCTD12 cannot bind to GB2 (Y902A). Data are means ± SD, $n = 7–12$. **, $p < 0.01$; t test.

may influence the ternary receptor/KCTD12/G-protein complex and vice versa, KCTD12 may influence S892 phosphorylation. We therefore addressed whether PKA regulates KCTD12-induced K^+ current desensitization. We found that PKA phosphorylation of S892 reduces KCTD12-induced K^+ current desensitization in heterologous cells and cultured hippocampal neurons. Mechanistically, S892 phosphorylation induces a conformational change in the $GABA_B$ receptor/KCTD12 complex that slows KCTD12-induced desensitization. Reciprocally, the binding of KCTD12 promotes phosphorylation of S892, consistent with previous data showing that KCTD12 stabilizes receptors at the cell surface [21].

2. Material and methods

2.1. Cell lines and cultures of hippocampal neurons

HEK293T and CHO-K1 cells were maintained in DMEM (Life Technologies, CA, US) supplemented with 10% FBS (GE Healthcare, Buckinghamshire, UK) at 37 °C with 5% CO_2. CHO-K1 cells stably expressing GB1 and GB2 subunits were cultured as described [22]. Cultured hippocampal neurons were prepared from wild-type (WT), KCTD12$^{-/-}$ [19,23] or GB2-S892A mice on embryonic day 16.5 as described [24].

2.2. Drugs

To activate $GABA_B$ receptors, baclofen (Cat.No. 417) was used at a final concentration of 100 μM. To activate PKA, 8-Br-cAMP (Cat.No. 1140) and forskolin (Cat.No. 1099) were used at a final concentration of 1 mM and 20 μM, respectively. To inhibit PKA, H89 (Cat.No. 2910) and protein kinase inhibitor-(14-22)-amide (PKI, Cat.No. 2546) were used at a final concentration of 2 μM or 10 μM, and 10 μM, respectively. All reagents were purchased from Tocris Bioscience, UK.

2.3. Generation of GB2-S892A knock-in mice

The gene targeting construct contained a neomycin resistance cassette (pRay-2; Genbank accession number U63120) flanked by two arms of Balb/c GB2 genomic DNA. The S892A mutation in exon 19 was introduced into the 3′ arm along with a diagnostic silent NheI restriction site. The targeting construct was electroporated into Balb/c embryonic stem cells. Neomycin resistant colonies were screened for homologous recombination by PCR, and positive clones confirmed by Southern blot. Positive ES cells were microinjected into C57BL/6 blastocysts. Chimeric males were crossed with female Balb/c mice to generate inbred Balb/c mice heterozygous for the S892A allele. The neomycin cassette in the S892A allele was then excised by mating heterozygous mice with Balb/c Cre-deleter mice [25]. Whole brain membranes were probed with the primary antibodies guinea-pig anti-GB2 (AB5394, 1:2000, Millipore, Darmstadt, Germany), rabbit anti-GB2-pS892 [13] (1:2000), rabbit anti-GB1 [26] (1:3000), mouse anti-β-III-tubulin (T8660, 1:1000, Sigma–Aldrich, MO, US). All animal experiments were subjected to institutional review and approved by the Veterinary Office of Basel-Stadt.

2.4. Molecular biology

The plasmids encoding HA-GB2, HA-GB2-Y902A, Myc-GB1b, Flag-GB1a, pRK6-GB1a-YFP, pcDNA3.1-GIRK1/2, Myc-KCTD12, pIRES-EGFP-KCTD12, Rluc-KCTD12 and Rluc-$G_{\beta1}$ were described earlier [19,21,27,28]. The HA-GB2-S892A and HA-GB2-S892E mutations were generated by replacing S892 with alanine or glutamate, respectively. Lipofectamine 2000 (Life Technologies, CA, US) was used for transient transfections. For electrophysiology we co-expressed green fluorescent protein (GFP) or pIRES-EGFP-KCTD12. The amount of DNA in the transfections was kept constant by supplementing with pCI plasmid DNA (Promega, WI, US).

2.5. Electrophysiology

CHO-K1 cells and cultured hippocampal neurons were plated onto Thermanox™ plastic coverslips (Thermo Scientific, MA, US) or poly-L-lysine (P9155, Sigma–Aldrich, MO, US) coated glass coverslips, respectively. Experiments with CHO-K1 cells and cultured hippocampal neurons were performed at room temperature 2–3 days or 1–2 weeks (DIV14-21) after transfection, respectively. Cells were continuously superfused with an extracellular solution (ECS) composed of (in mM): 145 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 25 glucose; pH 7.3, 323 mosm. Neurons were superfused with ECS supplemented with (in μM): 10 DNQX, 0.5 TTX, 100 picrotoxin, 5 D-CPP. Patch pipettes had resistances between 3 and 4 MΩ when filled with intracellular solution (ICS) composed of (in mM): 107.5 potassium gluconate, 32.5 KCl, 10 HEPES, 5 EGTA, 4 MgATP, 0.6 NaGTP, 10 Tris-phosphocreatine; pH 7.2, 295 mosm. To activate PKA the ICS was supplemented with 250 μM 8-Br-cAMP. Series resistance (<5 MΩ) was compensated by 80%. $GABA_B$ responses were evoked by fast application of baclofen and recorded with an Axopatch 200B patch-clamp amplifier; filtering and sampling frequencies were set to 1 kHz and 5 kHz, respectively [29]. Desensitization time constants were derived from bi-exponential fits to the decay phase of GIRK currents during agonist application. Curve fitting and data analysis were done with pClamp10 software (Molecular Devices, CA, US). Data are given as mean ± SD. Pharmacological treatments were carried out after DIV15 (neurons) or 48 h after transfection (CHO-K1 cells). Before recording, hippocampal neurons or CHO-K1 cells were pre-incubated for 30 min with 8-Br-cAMP, forskolin, PKI and H89 in the ECS.

2.6. Co-immunoprecipitation experiments and drug treatments

Forty-eight hours after transfection, HEK293T cells were starved for 4 h in DMEM w/o FCS prior to incubation for 30 min with 8-Br-cAMP. Cells were harvested, washed once with ice-cold PBS, and subsequently lysed in NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris/HCl, pH 7.4) supplemented with complete EDTA-free protease inhibitor mixture (Hoffmann-La Roche, Basel, Switzerland) and phosphatase inhibitors 2 and 3 (P5726 and P0044, Sigma–Aldrich, MO, US). After rotation for 10 min at 4 °C, the lysates were cleared by centrifugation at 1000 × *g* for 10 min at 4 °C. Lysates were directly used for Western blot analysis or immunoprecipitated with anti-Rluc antibody (MAB4410, Millipore, Darmstadt, Germany) coupled to Dynabeads® Protein-G (10004D, Life Technologies, CA, US). Lysates and immunoprecipitates were resolved using standard SDS–PAGE and probed with the primary antibodies guinea-pig anti-GB2 (AB5394, 1:2000, Millipore, Darmstadt, Germany) or guinea-pig anti-KCTD12 [23] (1:1000) and peroxidase-coupled secondary antibodies donkey anti-guinea pig (A7289, 1:10,000, Sigma–Aldrich, MO, US). The chemiluminescence detection kit (Thermo Scientific, MA, US) was used for visualization. The band intensity on Western blots was quantified from non-saturated images using a Fusion FX Chemiluminescence System (Vilber Lourmat, Witec AG, Lucerne, Switzerland). GB2 protein sometimes runs as two bands on SDS–PAGE, depending on the acrylamide concentration of the gel, the voltage applied across the gel and the electrophoresis time.

Drug treatments of HEK293T cells and cultured hippocampal neurons were performed 48 h after transfection or at DIV14, respectively. HEK293T cells were incubated with 8-Br-cAMP for 30 min. Cultured hippocampal neurons were incubated with H89 for 2 h. Lysates were probed with the primary antibodies

guinea-pig anti-GB2 (AB5394, 1:2000, Millipore, Darmstadt, Germany) or rabbit anti-GB2-pS892 [13] (1:750) and peroxidase-coupled secondary antibodies donkey anti-guinea pig (A7289, 1:10,000, Sigma–Aldrich, MO, US) and donkey anti-rabbit (NA9340, 1:10,000, GE Healthcare, Buckinghamshire, UK). β-III-Tubulin (T8660, 1:10,000, Sigma–Aldrich, MO, US; peroxidase-coupled secondary antibody sheep anti-mouse, NA931, 1:10,000, GE Healthcare, Buckinghamshire, UK) was used to control for loading and to normalize GB2 and phosphorylated GB2 protein levels in neurons.

2.7. BRET measurements

BRET measurements were performed as described [19,21]. CHO-K1 cells stably expressing GB1 and GB2 subunits and HEK293T cells were transiently transfected with plasmids encoding Rluc and YFP fusion proteins and distributed into 96-well microplates (Greiner Bio-One, Kremsmünster, Austria; coated with poly-L-ornithine hydrobromide; P3655, Sigma–Aldrich, MO, US) at a density of 50,000 and 100,000 cells/well, respectively. Twenty-four hours after transfection, cells were starved for 4 h in DMEM w/o FCS. After washing, coelenterazine h (5 μM, NanoLight Technologies, AZ, US) was added with or without 8-Br-cAMP for 30 min. Luminescence and fluorescence signals were detected sequentially using an Infinite® F500 Microplate Reader (Tecan, Männedorf, Switzerland). Baclofen was added 5 min prior measurement. Each data point represents a technical quadruplicate.

2.8. Data analysis

All data are presented as mean ± SD. Statistical significance was assessed using unpaired Student's *t* test, one-way ANOVA followed by Dunnett's multiple comparison test or two-way ANOVA followed by Sidak's multiple comparison test, using Prism 5.04 software (GraphPad, CA, US). BRET data were analyzed and fitted using a "one-site specific binding" equation (GraphPad, CA, US).

3. Results

3.1. S892 phosphorylation slows KCTD12-induced K^+ current desensitization

To investigate the effects of S892 phosphorylation on KCTD12-induced desensitization we performed whole-cell patch-clamp recordings from CHO-K1 cells expressing KCTD12, GIRK channels (GIRK1/2) and GB1b together with GB2-WT or the GB2 mutants S892A (phospho-deficient), S892E (phospho-mimetic) and Y902A (deficient in KCTD12-binding). Applications of the $GABA_B$ receptor agonist baclofen for 60 s elicited K^+ currents with much more pronounced desensitization in the presence (91.0 ± 3.4%; n = 14; Fig. 1B) than in the absence of KCTD12 (26.0 ± 7.5%; n = 10; Fig. 1C), as reported earlier [18]. Approximation of the time-course of the KCTD12-induced desensitization by a double exponential function yielded time constants of 1.6 ± 0.4 s (τ_1; relative amplitude 47.5 ± 15.3%) and 12.9 ± 2.1 s (τ_2; Fig. 1B) [18,19]. Activation of PKA by pre-incubation of cells with 8-Br-cAMP for 30 min significantly slowed both components

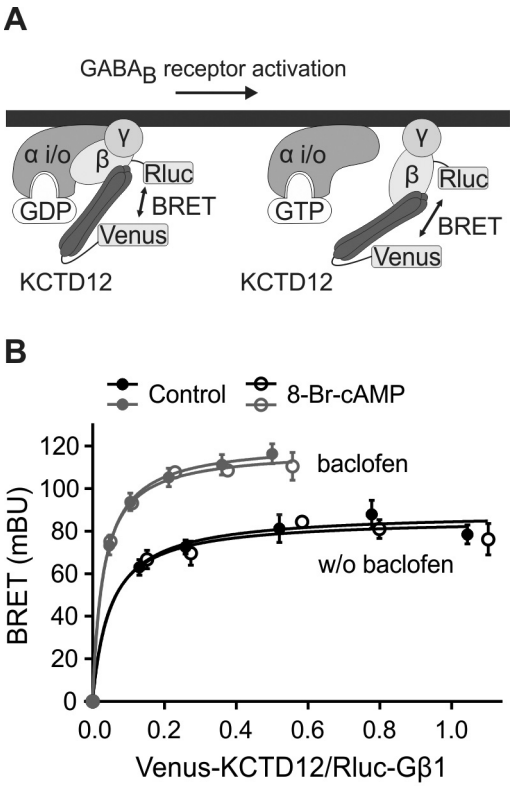

Fig. 3. Binding of KCTD12 to the G-protein βγ subunits is modulated by receptor activation but not by PKA activation. (A) Scheme of the BRET Rluc-$G_{\beta1}$ donor and Venus-KCTD12 acceptor pair. (B) BRET donor saturation curves from CHO-K1 cells stably expressing GB1 and GB2 and transiently expressing fixed amounts of Rluc-$G_{\beta1}$ and $G_{\gamma2}$ together with increasing amounts of Venus-KCTD12. Following receptor activation with baclofen (100 μM, 5 min pre-incubation; grey traces) the BRET curves were shifted towards higher $BRET_{max}$ values. Activation of PKA with 8-Br-cAMP (1 mM, 30 min pre-incubation; open circles) had no effect on the BRET curves. BRET is expressed as milli BRET units (mBU) determined as net BRET × 1000. Data points are means ± SD of technical quadruplicates of a representative experiment, n = 3.

Table 1
Parameters from BRET saturation curves with Rluc-KCTD12 and GB1a-YFP. BRET donor saturation assays were performed in HEK293T cells transfected with fixed amounts of GB2-WT or GB2-S892A, Rluc-KCTD12 (donor) and increasing amounts of GB1a-YFP (acceptor) with and without activation of PKA by pre-incubation with 8-Br-cAMP for 30 min. The $BRET_{max}$ is the maximal BRET signal obtained for a given donor/acceptor pair and the $BRET_{50}$ corresponds to the amount of acceptor needed to obtain 50% of the $BRET_{max}$. Data are presented as the mean ± SD of the indicated number of independent experiments and were derived from data as presented in Fig. 2.

GB2 constructs	$BRET_{max}$	SD	$BRET_{50}$	SD	n
GB2-WT	119.0	9.2	0.0037	0.0014	7
GB2-WT, 8-Br-cAMP	132.1*	12.4	0.0042	0.0013	7
GB2-S892A	112.8	3.6	0.0034	0.0008	4
GB2-S892A, 8-Br-cAMP	111.8	4.7	0.0033	0.0004	4

* $p < 0.05$; compared to GB2-WT; t test.

Table 2
Parameters from BRET saturation curves with Rluc-$G_{\beta1}$ and Venus-KCTD12. BRET donor saturation assays were performed in CHO-K1 cells stably expressing $GABA_B$ receptors and transiently expressing fixed amounts of Rluc-$G_{\beta1}$ (donor) and $G_{\gamma2}$ and increasing amounts of Venus-KCTD12 (acceptor). To activate $GABA_B$ receptors, cells were pre-incubated with baclofen for 5 min. To activate PKA, cells were pre-incubated with 8-Br-cAMP for 30 min. The $BRET_{max}$ is the maximal BRET signal obtained for a given donor/acceptor pair and the $BRET_{50}$ corresponds to the amount of acceptor needed to obtain 50% of the $BRET_{max}$. Data are presented as the mean ± SD of the indicated number of independent experiments and were derived from data as presented in Fig. 3.

$GABA_B$ receptor activation	$BRET_{max}$	SD	$BRET_{50}$	SD	n
Control, no baclofen	97.9	11.4	0.0490	0.0321	3
8-Br-cAMP, no baclofen	94.3	7.1	0.0517	0.0338	3
Control, baclofen	131.1*	21.9	0.0372	0.0125	3
8-Br-cAMP, baclofen	130.0*	21.9	0.0459	0.0264	3

* $p < 0.05$; compared to control, no baclofen; compared to 8-Br-cAMP, no baclofen; t test.

of desensitization (τ_1 = 3.7 ± 1.8 s, relative amplitude 44.2 ± 14.5%; τ_2 = 20.8 ± 2.2 s; $p < 0.01$ and $p < 0.001$; *t* test; mean time constant τ shown in Fig. 1G). In contrast, 8-Br-cAMP did not significantly influence $GABA_B$-activated K^+ currents in the absence of KCTD12 showing that PKA selectively modulates KCTD12-induced fast desensitization (Fig. 1C and G). Inhibition of PKA using H89 accelerated desensitization, suggestive of tonic PKA activity in CHO-K1 cells (τ_1 = 1.1 ± 0.3 s, relative amplitude 47.8 ± 19.5%; τ_2 = 7.2 ± 1.2 s, $p < 0.05$ and $p < 0.01$; *t* test). 8-Br-cAMP did not influence desensitization when expressing GB2-S892A or GB2-S892E instead of GB2-WT (Fig. 1D, E and G) thus implicating S892 in PKA modulation. Fast desensitization was not influenced by PKA when expressing GB2-Y902A (Fig. 1F, G). Of note, fast desensitization was already significantly slowed (increased τ) with GB2-Y902A compared to GB2-WT in the absence of 8-Br-cAMP (Fig. 1G). This confirms that assembly of receptors with KCTD12 is required for PKA regulation of fast desensitization.

Inhibition of PKA accelerates fast desensitization, suggestive of basal S892 phosphorylation. We therefore investigated whether repetitive activation of $GABA_B$ receptors, which reduces PKA-dependent phosphorylation of S892 [13], accelerates the KCTD12-induced desensitization. We recorded K^+ currents in response to repeated baclofen application for 60 s in 5-min intervals. In experiments with GB2-WT, but not with GB2-S892A, GB2-S892E or GB2-Y902A, desensitization of the 3rd K^+ current response was

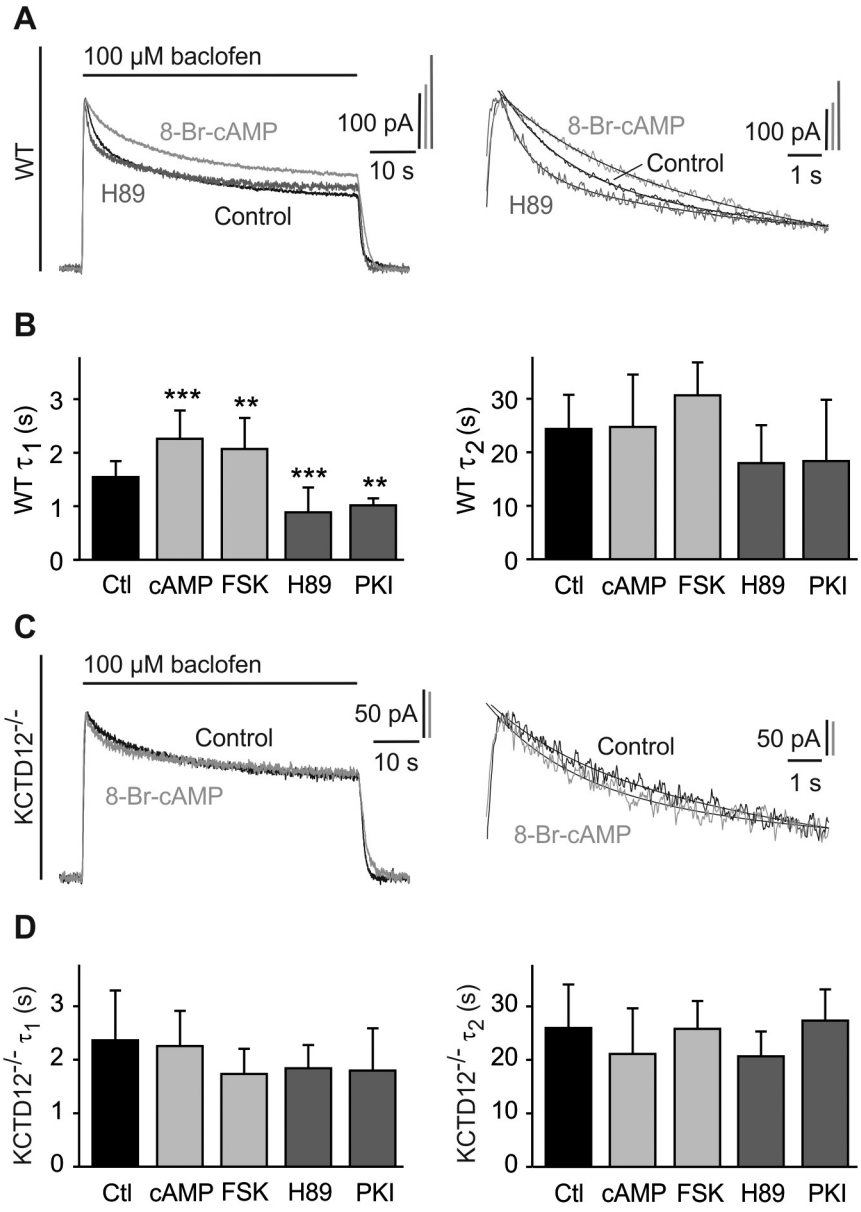

Fig. 4. PKA activation slows fast desensitization of baclofen-activated K^+ currents in cultured hippocampal neurons of WT but not of KCTD12$^{-/-}$ mice. (A) Representative baclofen-activated K^+ currents recorded at −50 mV from WT hippocampal neurons. PKA activity was modulated by pre-incubation for 30 min with 8-Br-cAMP (1 mM; bright grey trace) or H89 (2 µM; dark grey trace). Controls represent recordings from untreated neurons (black trace). The desensitization time constants τ_1 and τ_2 were derived from double-exponential fits to the decay phase of K^+ currents during baclofen application (enlarged on the right). (B) Bar graph summarizing the time constants τ_1 and τ_2 of baclofen-induced K^+ current desensitization for the indicated treatments. Data are means ± SD, n = 7–35. **, $p < 0.01$; ***, $p < 0.001$; Dunnett's multiple comparison test, compared to Ctl. Ctl, control; FSK, forskolin; cAMP, 8-Br-cAMP. (C) Representative baclofen-activated K^+ current responses from hippocampal KCTD12$^{-/-}$ neurons pre-incubated with 8-Br-cAMP (grey trace) or untreated KCTD12$^{-/-}$ neurons (control, black trace) as in (A). Traces were fitted as in (A). (D) Bar graph summarizing the time constants τ_1 and τ_2 of baclofen-induced K^+ current desensitization for the indicated treatments. Data are means ± SD, n = 5-17, Dunnett's multiple comparison test, compared to Ctl.

significantly faster by 16.4 ± 15.5% compared to the first response (Fig. 1H, I). In summary, the results show that KCTD12-induced desensitization of $GABA_B$-activated K^+ currents is regulated by receptor activity and PKA phosphorylation of S892. Repeated activation of $GABA_B$ receptors results in a sharpening of the receptor response due to a receptor activity-dependent reduction in S892 phosphorylation.

3.2. S892 phosphorylation rearranges the receptor/KCTD12 complex

It is conceivable that dissociation of KCTD12 from the receptor underlies the slowing of desensitization in response to PKA phosphorylation of S892. To test this hypothesis, we used a BRET assay monitoring the interaction of KCTD12 with $GABA_B$ receptors in living cells [21]. Binding of the donor fusion protein Rluc-KCTD12 to GB2 allows for specific BRET to the acceptor fusion protein GB1a-YFP (Fig. 2A). Expression of increasing amounts of GB1a-YFP with a fixed amount of Rluc-KCTD12 and GB2-WT resulted in hyperbolic BRET donor saturation curves, consistent with a specific interaction between KCTD12 and $GABA_B$ receptors (Fig. 2B, left). As a control, very low non-specific linear BRET was observed between Rluc-KCTD12 and GB1a-YFP in experiments with GB2-Y902A. When GB2-expressing cells were pre-incubated with 8-Br-cAMP for 30 min, we still observed a specific BRET donor saturation curve. This demonstrates that KCTD12 does not dissociate from the receptor upon receptor phosphorylation. However, compared to BRET experiments with untreated cells, the donor saturation curves in response to PKA activation were shifted towards higher maximal BRET ($BRET_{max}$) values (Fig. 2B, left, and Table 1). Given that similar amounts of BRET acceptor were required to reach 50% of the $BRET_{max}$ signal ($BRET_{50}$), the increase in $BRET_{max}$ most likely reflects a conformational rearrangement in the $GABA_B$ receptor/KCTD12 complex and not a change in the relative affinity of the proteins for one another or increased levels of $GABA_B$ receptors at the plasma membrane. Western blot analysis demonstrated that 8-Br-cAMP treatment significantly increased phosphorylation of S892 without altering total GB2 expression levels (Fig. 2C). We next acquired BRET donor saturation curves for Rluc-KCTD12 and GB1a-YFP in cells expressing GB2-S892A. As expected, we obtained hyperbolic BRET donor saturation curves demonstrating a specific interaction between KCTD12 and $GABA_B$ receptors assembled with GB2-S892A (Fig. 2B, right). However, 8-Br-cAMP did not alter the $BRET_{max}$ and $BRET_{50}$ values showing that PKA rearranges the receptor/KCTD12 complex through phosphorylation of S892 (Fig. 2B, right, and Table 1).

The finding that KCTD12 remains associated with $GABA_B$ receptors when activating PKA was further confirmed in co-immunoprecipitation experiments showing that similar amounts of GB2 are associated with KCTD12 in the presence and absence of 8-Br-cAMP (8-Br-cAMP: 102.8 ± 15.9%, $p > 0.05$ normalized to without 8-Br-cAMP; t test; $n = 5$; Fig. 2D). Co-immunoprecipitation experiments with receptors assembled with GB2-S892A or GB2-S892E confirmed that phosphorylation of S892 in GB2 is neither required nor prohibitive for binding of KCTD12 (Fig. 2E).

3.3. PKA does not impair dynamic binding of KCTD12 to the G-proteins

Since PKA induces a conformational rearrangement in the receptor/KCTD12 complex, we next investigated whether PKA also influences the interaction of KCTD12 with the G-protein [19]. This can be monitored by BRET between Rluc-$G_{\beta 1}$ and Venus-KCTD12 in transfected CHO-K1 cells stably expressing GB1 and GB2 subunits (Fig. 3A). In agreement with KCTD12 dynamically interacting with the G-protein [19], the BRET donor saturation curves were shifted towards higher $BRET_{max}$ values following activation of $GABA_B$ receptors with baclofen (Fig. 3B and Table 2). This agrees with an activity-dependent conformational rearrangement of KCTD12 at the G-protein reported earlier [19]. However, 8-Br-cAMP did not alter the BRET donor saturation curve between Rluc-$G_{\beta 1}$ and Venus-KCTD12 irrespective of whether $GABA_B$ receptors were activated with baclofen or not (Fig. 3B and Table 2). These data therefore demonstrate that activation of PKA does not alter the interaction between KCTD12 and the G-protein in a measurable way.

3.4. S892 phosphorylation slows KCTD12-induced K^+ current desensitization in cultured hippocampal neurons

We next investigated whether PKA modulates the desensitization of $GABA_B$-activated K^+ currents in cultured hippocampal neurons, which are known to express KCTD12 [18,19,23]. With WT neurons, K^+ currents elicited by 60-s long applications of baclofen showed a steady-state desensitization of 53.3 ± 9.3% ($n = 41$, Fig. 4A). The time course of desensitization was approximated by a double exponential function with time constants of 1.5 ± 0.3 s (τ_1) and 24.4 ± 6.4 s (τ_2) (Fig. 4B), values that are similar as in earlier experiments [18,19]. Activation of PKA with 8-Br-cAMP or forskolin significantly increased the fast component τ_1 of the desensitization, while inhibition of PKA with H89 or PKI had the opposite effect (Fig. 4B). Neither activation nor inhibition of PKA had a significant effect on the slow component τ_2 of the desensitization (Fig. 4B). The relative contribution of the fast and slow components (τ_1 and τ_2, respectively) to the desensitization did not change with any of the treatments (τ_1 of control: 46.5 ± 10.0%; cAMP: 36.3 ± 15.4%; FSK: 40.3 ± 17.8%; H89: 55.7 ± 12.2%; PKI: 47.7 ± 7.7%; $p > 0.05$; Dunnett's multiple comparison test). These results reveal that PKA activity specifically influences the fast component of baclofen-induced K^+ current desensitization in WT neurons.

In cultured hippocampal neurons of $KCTD12^{-/-}$ mice [19] τ_1 but not τ_2 was significantly increased compared to WT mice (τ_1 of WT: 1.5 ± 0.3 s; $KCTD12^{-/-}$: 2.4 ± 0.9 s; $p < 0.001$; τ_2 of WT: 24.4 ± 6.4 s; $KCTD12^{-/-}$: 26.0 ± 8.1 s; $p > 0.05$; t test; compare Fig. 4B and D). Neither activation nor inhibition of PKA had a significant effect on the desensitization of baclofen-induced K^+ currents in $KCTD12^{-/-}$ neurons (Fig. 4D). These data support that the PKA-modulated fast component of K^+ current desensitization in neurons depends on KCTD12.

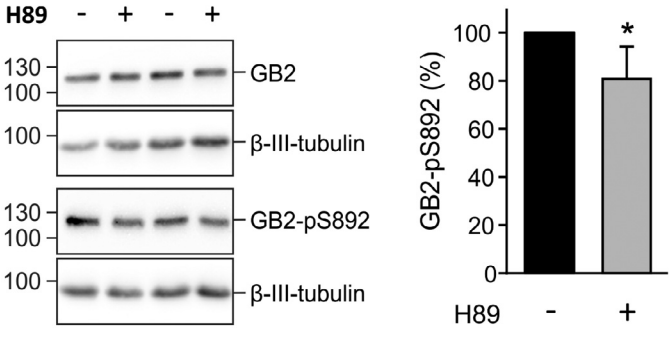

Fig. 5. Basal phosphorylation of S892 is decreased by inhibiting PKA in hippocampal neurons of WT mice. Western blot of cultured neurons in the presence and absence of H89 (10 μM, 2 h pre-incubation; left). Inhibition of PKA does not affect the amount of GB2 protein but decreases the amount of S892 phosphorylation (GB2-pS892). β-III-Tubulin was used as a loading control. Bar graph summarizing the amount of phosphorylated S892 (GB2-pS892 in %; right). The total amount of GB2 and GB2-pS892 were normalized to β-III-tubulin on the same blot. Data are means ± SD, $n = 6$. *, $p < 0.05$; t test.

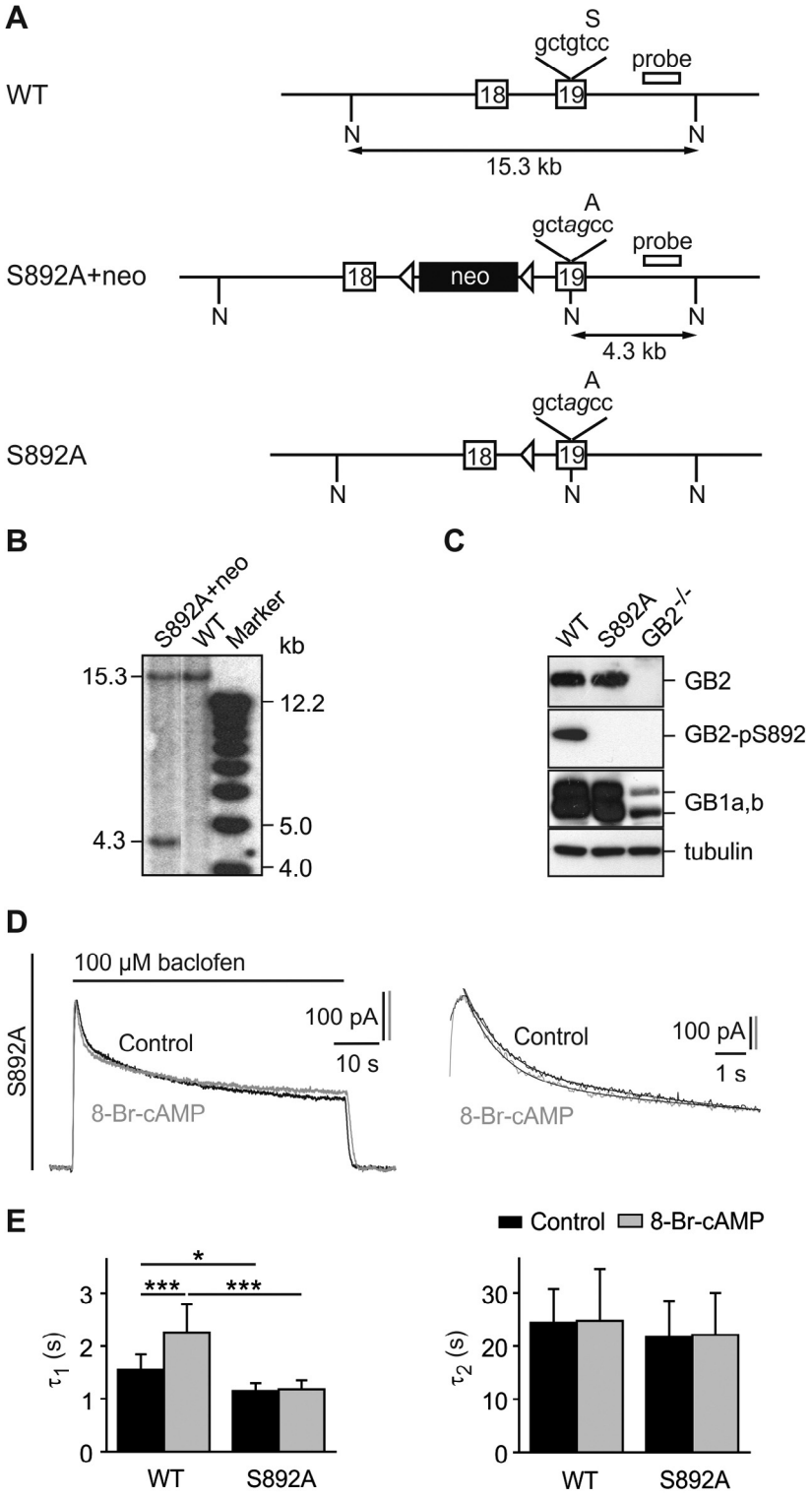

Fig. 6. Lack of S892 phosphorylation in S892A knock-in mice accelerates fast desensitization of $GABA_B$-activated K^+ currents. (A) WT and mutated GB2 alleles. The S892 to alanine mutation (tcc → gcc) and a silent diagnostic NheI restriction site (gctagc) were introduced into exon 19 using homologous recombination in Balb/c embryonic stem (ES) cells. Mutated nucleotides are shown in italic. A neomycin marker (neo) flanked by loxP sites (arrowheads) was used for selection of ES cells. Correctly targeted ES cells (S892A + neo allele) were injected into C57BL/6 blastocysts. A founder mouse was crossed with a Balb/c mouse expressing Cre-recombinase to excise the neomycin cassette, leaving one loxP site behind (S892A allele). The hybridization probe used in the Southern blot in (B) is indicated. A, alanine; N, NheI restriction sites; S, serine. (B) Southern blot of NheI cut genomic DNA from correctly targeted ES cells. The probe labels a 15.3 kb fragment for the WT allele and a 4.3 kb fragment for the S892A + neo allele. (C) Western blot analysis of brain extracts showing that S892A mice express normal levels of GB2, GB1a and GB1b proteins. S892 was phosphorylated in brain extracts of WT but not S892A mice as shown with an antibody specific for phosphorylated S892 (GB2-pS892). Brain extracts of GB2-deficient mice ($GB2^{-/-}$) [38] confirm the specificity of the GB2 and GB2-pS892 antibodies. β-III-Tubulin was used as a loading control. (D) Representative $GABA_B$-activated K^+ currents recorded at −50 mV in response to baclofen application (100 µM) from cultured hippocampal neurons of S892A mice. PKA was activated by pre-incubation for 30 min with 8-Br-cAMP (1 mM; grey trace). Controls represent recordings from untreated neurons (black trace). The desensitization time constants τ_1 and τ_2 were derived from double-exponential fits to the decay phase of K^+ currents during baclofen application (enlarged on the right). (E) Bar graph summarizing the time constants τ_1 and τ_2 of baclofen-induced K^+ current desensitization in WT and S892A neurons. Data are means ± SD, $n = 5–7$. *, $p < 0.05$; ***, $p < 0.001$; Sidak's multiple comparison test.

3.5. Lack of S892 phosphorylation in S892A knock-in mice accelerates fast desensitization of K^+ currents and prevents regulation by PKA

PKA inhibition with H89 or PKI accelerates fast desensitization of K^+ currents in WT hippocampal neurons. Endogenous PKA activity must therefore provide a high level of basal S892 phosphorylation. Indeed, Western blot analysis of hippocampal neurons revealed that GB2 is highly phosphorylated at S892 (Fig. 5), which is reduced by inhibition of PKA with H89. We next addressed whether phosphorylation of S892 in GB2 is essential for PKA effects on baclofen-induced K^+ current desensitization in neurons. We generated S892A knock-in mice carrying a S892 to alanine mutation in the GB2 gene using standard gene targeting techniques (Fig. 6A and B). S892A mice display no overt behavioral abnormalities. Western blot analysis revealed similar levels of GB1a, GB1b and GB2 protein in S892A and WT brain extracts (Fig. 6C). Recordings of baclofen-induced K^+ currents from cultured hippocampal neurons revealed that the desensitization was significantly faster in S892A compared to WT neurons (Fig. 6D and E). Activation of PKA with 8-Br-cAMP did not significantly increase τ_1 of the desensitization in S892A neurons, in contrast to control WT neurons (Fig. 6E). The τ_2 of the desensitization was similar in both genotypes and did not change upon activation of PKA with 8-Br-cAMP (Fig. 6E). The lack of PKA effect in S892A neurons indicates that S892 phosphorylation is mandatory for PKA-mediated attenuation of fast desensitization. In summary, our results show that basal PKA-mediated phosphorylation of S892 slows KCTD12-induced desensitization in neurons.

3.6. Assembly of KCTD12 with $GABA_B$ receptors increases S892 phosphorylation

We additionally investigated whether assembly of $GABA_B$ receptors with KCTD12 influences S892 phosphorylation. We found that co-expression of KCTD12 with $GABA_B$ receptors in HEK293T cells increases phosphorylation of S892 in WT but not in GB2-Y902A receptors (Fig. 7). These data reveal that binding of KCTD12 and phosphorylation of S892 influence each other.

4. Discussion

4.1. Slow and fast mechanisms of homologous desensitization of $GABA_B$ receptor responses influence each other

A slow form of homologous desensitization of $GABA_B$ receptor responses relies on the activity-dependent decrease of S892 phosphorylation in GB2 by PKA [13,14]. Here we show that phosphorylation of S892 by PKA slows KCTD12-induced desensitization, a faster form of desensitization that is induced at the G-protein rather than at the receptor. We observed a high basal level of S892 phosphorylation in hippocampal neurons, consistent with earlier reports [13,30]. Accordingly, KCTD12-induced current desensitization is significantly attenuated in WT hippocampal neurons compared to GB2-S892A neurons. This may to some extent explain the moderate desensitization of $GABA_B$-activated K^+ currents in hippocampal neurons, despite a high level of KCTD12 expression [19]. Conversely, KCTD12 promotes S892 phosphorylation, which may explain why KCTD12 stabilizes receptors at the cell surface and increases the magnitude of $GABA_B$ receptor signaling [21]. These results indicate that the mechanism of fast and slow desensitization influence each other.

4.2. Interplay of slow and fast desensitization mechanisms

KCTD12 binds to GB2 in close proximity of the PKA phosphorylation-site S892. In principle, phosphorylation of S892

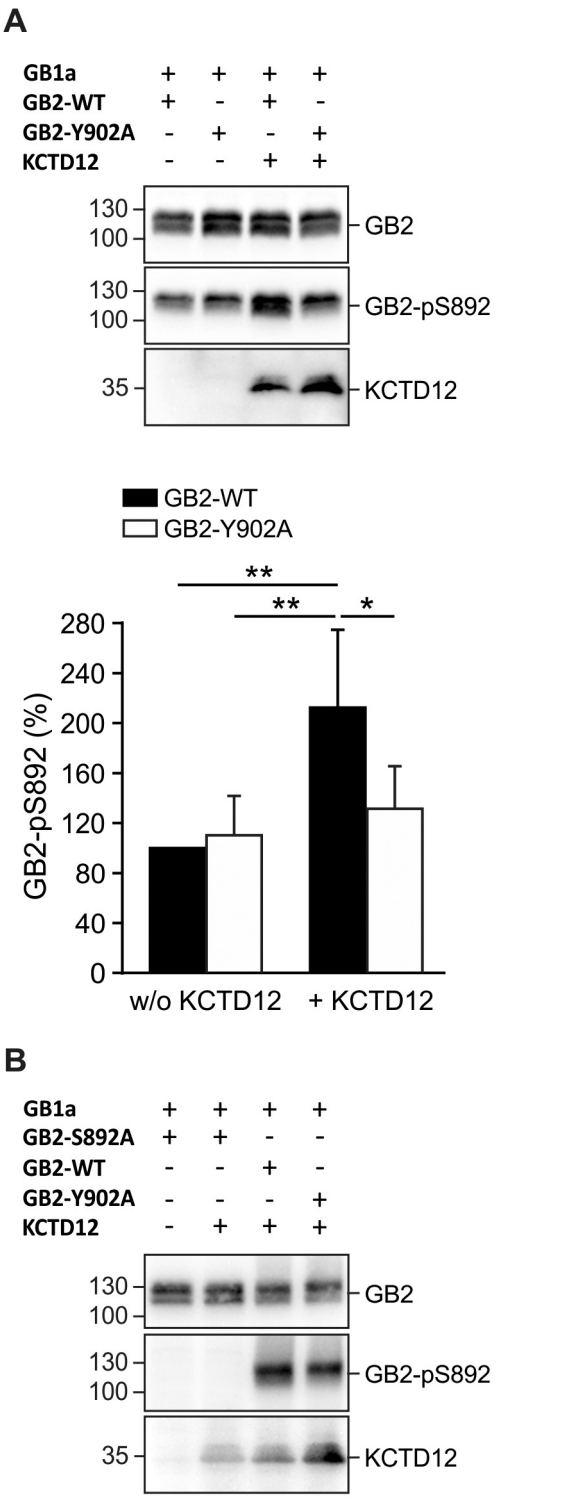

Fig. 7. Binding of KCTD12 to $GABA_B$ receptors increases S892 phosphorylation. (A) Lysates of HEK293T cells expressing GB1a and either GB2-WT or GB2-Y902A in the presence and absence of KCTD12 (up). S892 phosphorylation (GB2-pS892) is increased in the presence of KCTD12 for GB2-WT, but not for GB2-Y902A. Blots are representative of five independent experiments. Bar graph summarizing the amount of phosphorylated S892 normalized to GB2 protein on the same blot (GB2-pS892 in %; bottom). Data are means ± SD, $n = 5$. *, $p < 0.05$; **, $p < 0.01$; Sidak's multiple comparison test. (B) Western blot of lysates from transfected HEK293T cells expressing the indicated proteins. Phosphorylation of S892 (GB2-pS892) is observed with GB2-WT and GB2-Y902A but not with GB2-S892A, showing that the pS892 antibody specifically detects phosphorylation of S892. Note increased phosphorylation of S892 in the presence of KCTD12 with WT receptors (GB2-WT) compared to mutant receptors that cannot bind KCTD12 (GB2-Y902A). Blots are representative of three independent experiments.

could facilitate unbinding of KCTD12 from the receptor, which would explain the slowing of fast desensitization. However, BRET analysis and co-immunoprecipitation experiments show that neither PKA activity nor the phospho-mimetic mutant GB2-S892E unbind KCTD12 from the receptor. This agrees with earlier findings showing that KCTD12 remains associated with the receptor during receptor activity [21]. However, BRET analysis revealed that PKA-mediated S892 phosphorylation induces a conformational change in the receptor/KCTD12 complex without measurably altering the interaction between KCTD12 and the G-protein. This suggests that S892 phosphorylation does not stabilize a receptor/KCTD12 conformation that prevents binding of KCTD12 to Gβγ [19]. Since GB2 and KCTD12 both bind to the G-protein the conformational rearrangement in the receptor/KCTD12 complex may reduce the rate of G-protein activation [19,27,31–33]. Because KCTD12-induced desensitization is activity-dependent [19], a reduction in the G-protein activation rate would slow fast desensitization.

Conversely, assembly of $GABA_B$ receptors with KCTD12 promotes S892 phosphorylation, which may induce a conformational rearrangement in the cytoplasmic domain of GB2 that makes S892 more accessible for phosphorylation by PKA. This agrees with earlier findings that show that receptors with KCTD12 are more stable at the cell surface [21].

Overall, the interplay of fast and slow mechanisms of homologous desensitization will have several effects on $GABA_B$ receptor-activated K^+ current responses. Assembly of receptors with KCTD12 will increase basal S892 phosphorylation and stabilize receptors at the cell surface [21]. Increased tonic S892 phosphorylation will attenuate KCTD12-induced fast desensitization. Conversely, prolonged exposure to agonists will reduce PKA activity and S892 phosphorylation. This will accelerate KCTD12-induced fast desensitization and promote slow desensitization due to receptor degradation. The interplay of the two mechanisms of desensitization underlies the sharpening of the K^+ current response observed in heterologous cells during repeated receptor activation. In addition to this homologous regulation of desensitization, it is possible that other GPCRs regulating cAMP/PKA signaling modulate $GABA_B$ receptor desensitization in a heterologous manner. Moreover, $GABA_B$ receptor desensitization may be regulated at S892 during neuronal processes in which PKA activity is altered, such as NMDA-mediated synaptic plasticity, learning and memory, seizures or during aging [34–37].

Acknowledgments

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2.3 Pharmacological characterization of $GABA_B$ receptor subtypes assembled with auxiliary KCTD subunits

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Pharmacological characterization of $GABA_B$ receptor subtypes assembled with auxiliary KCTD subunits

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ABSTRACT

$GABA_B$ receptors ($GABA_BRs$) are considered promising drug targets for the treatment of mental health disorders. $GABA_BRs$ are obligate heteromers of principal $GABA_{B1}$ and $GABA_{B2}$ subunits. $GABA_BRs$ can additionally associate with auxiliary KCTD8, 12, 12b and 16 subunits, which also bind the G-protein and differentially regulate G-protein signaling. It is unknown whether the KCTDs allosterically influence pharmacological properties of $GABA_BRs$. Here we show that KCTD8 and KCTD16 slightly but significantly increase GABA affinity at recombinant receptors. However, KCTDs clearly do not account for the 10-fold higher GABA affinity of native compared to recombinant $GABA_BRs$. The positive allosteric modulator (PAM) GS39783, which binds to $GABA_{B2}$, increases both potency and efficacy of GABA-mediated G-protein activation ($[^{35}S]GTP\gamma S$ binding, BRET between G-protein subunits), irrespective of whether KCTDs are present or not. Of note, the increase in efficacy was significantly larger in the presence of KCTD8, which likely is the consequence of a reduced tonic G-protein activation in the combined presence of KCTD8 and $GABA_BRs$. We recorded Kir3 currents to study the effects of GS39783 on receptor-activated G-protein $\beta\gamma$-signaling. In transfected CHO cells and cultured hippocampal neurons GS39783 increased Kir3 current amplitudes activated by 1 μM of baclofen in the absence and presence of KCTDs. Our data show that auxiliary KCTD subunits exert marginal allosteric influences on principal $GABA_BR$ subunits. PAMs at principal subunits will therefore not be selective for receptor subtypes owing to KCTD subunits. However, PAMs can differentially modulate the responses of receptor subtypes because the KCTDs differentially regulate G-protein signaling.

This article is part of a Special Issue entitled 'GABAergic signaling'.

1. Introduction

$GABA_BRs$ are the G-protein-coupled receptors (GPCRs) for GABA, the main inhibitory neurotransmitter in the mammalian brain. They play important roles in regulating neuronal excitability and have been implicated in a variety of neurological and psychiatric disorders (Gassmann and Bettler, 2012). $GABA_BRs$ activate $G\alpha_{i/o}$-type G-proteins that inhibit adenylyl cyclase via $G\alpha_{i/o}$ and gate ion channels via $G\beta\gamma$. It is well established that presynaptic $GABA_BRs$ inhibit voltage-gated Ca^{2+} channels and neurotransmitter release. Postsynaptic $GABA_BRs$ activate inwardly rectifying K^+ channels (Kir3 channels) that generate slow inhibitory postsynaptic potentials and inhibit neuronal excitability by local shunting.

$GABA_BRs$ are heteromeric complexes of $GABA_{B1}$ and $GABA_{B2}$ subunits (Gassmann and Bettler, 2012). Two predominant $GABA_{B1}$ subunit isoforms exist, $GABA_{B1a}$ and $GABA_{B1b}$, which constitute heteromeric $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors with similar

Abbreviations: 3KO, *Kctd8/12/16*$^{-/-}$ triple knockout mice; A_1, adenosine receptor 1; ANOVA, analysis of variance; BRET, bioluminescence resonance energy transfer; CHO cells, Chinese hamster ovary K1 cells; CHO-$GABA_B$, CHO cells stably expressing $GABA_{B(1b,2)}$ receptors; CGP7930, 2,6-Di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol; ΔBRET, changes in BRET; EC_{50}, half maximal effective concentration; EGFP, enhanced green fluorescent protein; E_{max}, maximum stimulatory effect; $GABA_BR$, $GABA_B$ receptor; GDP, guanosine 5'-diphosphate; GPCR, G-protein-coupled receptor; GS39783, N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine; $[^{35}S]GTP\gamma S$, $[^{35}S]$guanosine 5′-O-(3-thio)triphosphate; HEK293T cells, Human Embryonic Kidney 293T cells; HIV-1, human immunodeficiency virus-1; IC_{50}, half maximal inhibitory concentration; KCTD, Potassium Channel Tetramerisation Domain; KH buffer, Krebs-Henseleit buffer; Kir, K^+ inwardly rectifying; mBU, milli BRET units; PAM, positive allosteric modulator; Rluc, *Renilla reniformis* luciferase.

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pharmacological and functional properties in heterologous cells (Brauner-Osborne and Krogsgaard-Larsen, 1999; Green et al., 2000; Kaupmann et al., 1998). $GABA_{B1}$ subunits contain the GABA binding-site while the $GABA_{B2}$ subunit couples to the G-protein. $GABA_{B2}$ allosterically induces a 10-fold increase in agonist binding-affinity at $GABA_{B1}$. However, for unknown reasons, recombinant $GABA_BRs$ still have a 10-fold lower agonist binding-affinity than the native receptors (Kaupmann et al., 1998). $GABA_{B1a}$ and $GABA_{B1b}$ target $GABA_BRs$ to pre- and postsynaptic compartments, respectively (Biermann et al., 2010; Vigot et al., 2006). We recently showed that $GABA_BRs$ can constitutively associate with homotetramers of K^+ channel tetramerization-domain (KCTD) containing proteins. $GABA_BRs$ assembled with KCTD8, 12, 12b and 16 constitute molecularly and functionally distinct receptor subtypes (Schwenk et al., 2010). The KCTDs are cytoplasmic proteins that simultaneously bind to $GABA_{B2}$ and the Gβγ subunits of the heterotrimeric G-protein (Turecek et al., 2014). Dual binding to receptor and G-protein allows the KCTDs to stabilize the G-protein/receptor interaction, which overcomes the slow diffusion-limited association of the G-protein with the receptor and accelerates G-protein signaling (Turecek et al., 2014). Furthermore, KCTD12 uncouples Gβγ from effector channels to induce a fast (within seconds) and pronounced current desensitization (Turecek et al., 2014). The prototypical synthetic $GABA_BR$ agonist baclofen is 3–7 fold more potent in activating Kir3 currents in the presence of KCTD proteins (Schwenk et al., 2010). Whether this increase in agonist potency in the presence of the KCTDs relates to an increase in agonist affinity at $GABA_{B1}$ or to activity-dependent effects at the G-protein, for example to an acceleration of the G-protein cycle, is unknown.

The ternary $GABA_BR$ complex assembled from $GABA_{B1}$, $GABA_{B2}$, KCTD and G-protein subunits offers ample opportunities for allosteric regulation. For example, it is well established that the orthosteric agonist binding-site in $GABA_{B1}$ is allosterically coupled to the G-protein binding-site in $GABA_{B2}$ (Hill et al., 1984; Pin et al., 2004). Likewise, PAMs (GS39783 and CGP7930) that bind to the $GABA_{B2}$ transmembrane domains strongly increase agonist binding-affinity at $GABA_{B1}$ (Binet et al., 2004; Dupuis et al., 2006; Mannoury la Cour et al., 2008; Urwyler et al., 2003). Whether association of the KCTDs with $GABA_{B2}$ and the G-protein also allosterically influences ligand-binding properties of $GABA_BRs$ is unknown.

Here we report that KCTD8 and KCTD16 slightly increase agonist binding-affinity at the receptor. However, this increase in agonist binding-affinity is clearly not reaching the still higher affinity of native $GABA_BRs$. Furthermore, GS39783 increases potency and efficacy of G-protein activation in the presence and absence of the KCTDs. In summary, our data indicate that the KCTDs exert marginal allosteric effects on the $GABA_{B1}$ and $GABA_{B2}$ protomers and primarily influence receptor signaling by acting at the G-protein. A conclusion from these experiments is that compounds acting at the $GABA_{B1}$ and $GABA_{B2}$ protomers will not allow to clearly distinguish receptor subtypes based on KCTD subunits. However, our data also show that by acting at the G-protein the KCTDs can to some extent differentially affect allosteric modulation of receptor signaling.

2. Materials and methods

2.1. Plasmids, cell culture and transfection procedure

Plasmids encoding Myc-$GABA_{B1b}$, Myc-$GABA_{B2}$, Flag-KCTDs, Kir3.1/3.2 concatamers, *Renilla reniformis* luciferase (Rluc)-tagged Gαo and Venus-Gγ2 were described previously (Ayoub et al., 2009; Ivankova et al., 2013; Schwenk et al., 2010). The plasmid expressing Flag-tagged Gβ2 was obtained from the Missouri S&T cDNA Resource Center and pEGFP-N1 from Clontech. The plasmid expressing Flag-tagged human adenosine receptor 1 (A_1) was a gift from Miriam Peeters (Center for Basic Metabolic Research, Copenhagen University).

Human Embryonic Kidney 293T (HEK293T) and Chinese hamster ovary K1 (CHO) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, glutamine-free, Invitrogen), supplemented with 10% FCS (Gibco, Life Technologies) in a humidified atmosphere of 5% CO_2 at 37 °C. CHO cells stably expressing $GABA_{B(1b,2)}$ (CHO-$GABA_B$) were described earlier (Urwyler et al., 2001) and maintained in DMEM supplemented with 500 μm L-glutamine (Sigma), 40 μg/ml L-proline (Sigma), 500 μg/ml geneticin (Roche), 250 μg/ml zeocin (Invitrogen) and 10% FCS under identical conditions as HEK293T and CHO cells.

Cultured hippocampal neurons were prepared as described previously (Brewer et al., 1993). Briefly, embryonic day 16.5 mouse hippocampi were dissected, digested with 0.25% trypsin (Invitrogen) in 1 × PBS solution (Gibco) for 15 min at 37 °C, dissociated by trituration, and plated on glass coverslips coated with 1 mg/ml poly-L-lysine hydrobromide (Sigma) in 0.1 M borate buffer (boric acid/sodium tetraborate). Neurons were seeded at high density (~550 cells/mm^2) and incubated for 14–21 days in a humidified atmosphere of 5% CO_2 at 37 °C. Cultures were grown in neurobasal medium (Gibco) supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine.

Lipofectamine 2000 (Invitrogen) was used for all transient transfections of HEK293T and CHO-$GABA_B$ cells. For electrophysiology experiments, CHO-$GABA_B$ cells were splitted at 80–90% confluency 24 h before transfection and plated onto plastic coverslips (Thermanox, Thermo Fisher Scientific) at a dilution of 1:5 in 35 mm dishes. CHO-$GABA_B$ cells were transfected using 2.5 μl/ml of Lipofectamine 2000 as well as 1.75 μg/ml of Kir3.1/3.2 concatamer, 0.625 μg/ml of KCTD constructs and 0.375 μg/ml of pEGFP-N1 to visualize transfected cells. After 5 h, the medium was exchanged and the cells were kept in the incubator for additional 48 h before being used for electrophysiological recordings.

2.2. Viral transduction of CHO cells

For stable expression of Flag-epitope tagged KCTD8 or KCTD12, CHO and CHO-$GABA_B$ cells, were transduced with lentiviral vectors as described earlier (Lois et al., 2002). Briefly, lentiviruses were produced by cotransfection of HEK293T cells with the lentiviral target vector FUW (Lois et al., 2002), the HIV-1 packaging vector Δ8.2 and the VSV-G envelope glycoprotein vector (Oliver Schlüter, European Neuroscience Institute, Göttingen). The self-inactivating and replication-deficient VSV-G pseudotyped viruses were concentrated by ultracentrifugation (74,000 *g*, 90 min, 4 °C) of the virus-containing supernatant.

Stable expression of Flag-KCTDs was achieved by transduction of low passage CHO cells using different concentrations of the virus-containing supernatant to obtain varying levels of KCTD expression. Pools of CHO cells with equal KCTD-expression levels were selected by Western blotting of cellular lysates and proteins were detected using an antibody against the Flag-epitope tag (Sigma).

2.3. Radioligand binding assay

Preparation of membranes from HEK293T cells for radioligand binding assays was performed as described in Galvez et al. (2000). Briefly, culture dishes were washed twice with ice-cold PBS, 10 mM of HEPES buffer, pH 7.4, was added to the plates and cells scraped off (BD Flacon). Crude membranes from approximately ten 15-cm cell culture dishes per point were collected and centrifuged (26,000 *g*, 20 min). The pellet was re-suspended in 10 ml HEPES buffer, homogenized using a glass-teflon homogenizer (10 strokes) and the suspension centrifuged (38,000 *g*, 20 min). The pellet was re-suspended in 2 ml of buffer and homogenized (20 strokes). Aliquots were frozen in liquid nitrogen and stored at −80 °C for 48 h.

Preparation of membranes from rat cortical neurons as well as mouse brains for radioligand binding assays was performed as described in Olpe et al. (1990). Briefly, animals of at least 8 weeks of age were decapitated, the brains removed, washed in ice-cold PBS and homogenized in 10 volumes of ice-cold 0.32 M sucrose, containing 4 mM HEPES, 1 m EDTA and 1 mM EGTA, using a glass-teflon homogenizer. Debris was removed at 1000 *g* (10 min) and membranes centrifuged at 26,000 *g* (15 min). The pellet was osmotically shocked by re-suspension in a 10-fold volume of ice-cold dH_2O and kept on ice for 1 h. The suspension was centrifuged at 38,000 *g* (20 min) and re-suspended in a 3-fold volume of dH_2O. Aliquots were frozen in liquid nitrogen and stored at −20 °C for 48 h. After thawing at room temperature, a 7-fold volume of Krebs-Henseleit (KH) buffer (pH 7.4) was added, containing 20 mM Tris-HCl, 118 mM NaCl, 5.6 mM glucose, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4 and 1.2 mM $MgSO_4$. Membranes were washed three times by centrifugation at 26,000 *g* (15 min), followed by re-suspension in KH buffer. The final pellet was re-suspended in a 5-fold volume of KH buffer. Aliquots (2 ml) were frozen and stored at −80 °C until further use.

On the day of the experiment, the membranes from frozen HEK293T cells or neurons were thawed, re-suspended in 10 ml of ice-cold dH_2O, and centrifuged at 26,000 *g* (15 min). The pellet was again re-suspended in 10 ml of ice-cold dH_2O and incubated for 1 h on ice. After one additional round of centrifugation at 26,000 *g* (15 min) the final pellet was re-suspended in assay buffer containing 50 mM Tris-HCl buffer (pH 7.7); 10 mM $MgCl_2$, 1.8 mM $CaCl_2$, 100 mM NaCl, 20 μg of membrane protein, 5 nM of the high-affinity $GABA_BR$ radioligand antagonist [^{3}H]CGP54626A (60 Ci/mmol, ANAWA AG, Wangen, Switzerland), in a final volume of 50 μl per point and in the absence and presence of competitor compound. Protein concentrations for the samples were measured with the Bradford assay method, using the Bio-Rad protein assay kit and bovine serum albumin as a standard.

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The reagents were incubated for 45 min at room temperature in 96-well polypropylene microplates (Greiner Bio-One) with mild shaking. They were subsequently filtered using 96-well Whatman GF/C glass fiber filters (Perkin Elmer), pre-soaked in assay buffer, using a Filtermate cell harvester (Perkin Elmer). After four washes with assay buffer, the Whatman filter fibers were dried for 1 h at 50 °C. 50 μl of scintillation fluid (Microscint 20, Perkin Elmer) was added, the plates were shaken for 1 h and thereafter counted using a Packard TopCount NXT (Perkin Elmer). GraphPad Prism 5.01 software (Graph Pad, San Diego, CA) was used for data analysis.

2.4. Western Blotting

For Western Blotting, HEK293T cells were harvested, washed in PBS, and subsequently lysed in a Nonidet P-40 buffer (100 mm NaCl, 1 mm EDTA, 0.5% Nonidet P-40, 20 mm Tris/HCl, pH 7.4) supplemented with complete EDTA-free protease inhibitor mixture (Roche). After rotation for 10 min at 4 °C, the lysates were cleared by centrifugation at 16,000 × *g* for 10 min at 4 °C. Lysates were thereafter resolved using standard SDS-PAGE and probed with the primary antibodies rabbit anti-Myc (Sigma), rabbit anti-$GABA_{B1}$ (Novartis) and guinea pig anti-$GABA_{B2}$ (Millipore). The antibody incubation was in 5% nonfat dry milk in PBS containing 0.1% Tween-20. The chemiluminescence detection kit (Pierce) was used for visualization.

2.5. [^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding assays with CHO cell membranes were performed as described (Urwyler et al., 2001). Briefly, CHO cells were grown to 80–90% confluency in 15-cm culture dishes. Culture dishes were washed twice with ice-cold PBS and subsequently treated as described for the radioligand binding assay with membranes of HEK293T cells with the following differences. The final pellet was re-suspended in assay buffer containing 50 mM Tris-HCl buffer (pH 7.7); 10 mM $MgCl_2$, 1.8 mM $CaCl_2$, 100 mM NaCl, 30 μM guanosine 5'-diphosphate (GDP, Sigma), 10 μg of membrane protein, 0.2 nM [^{35}S]GTPγS (Perkin Elmer), in the absence or presence of GS39783 (10 μM, dissolved in DMSO, a kind gift from K. Kaupmann, Novartis), in a final volume of 200 μl per point. Non-specific binding was measured in the presence of unlabeled GTPγS (10 μM, Sigma). To determine basal [^{35}S]GTPγS binding we used 1 μM of GDP in the assay buffer. Because maximal binding with GABA alone differed between experiments, the data were normalized to the maximal effect (E_{max}) of saturating concentrations of GABA in the absence of GS39783 (100%) and basal activity without GABA (0%).

For [^{35}S]GTPγS binding assays with mouse brain membranes, mice of at least 8 weeks of age were decapitated, the brains removed and subsequently processed as described for the radioligand binding assay with brain membranes. On the day of the experiment, the frozen membranes were thawed, homogenized in 10 ml ice-cold assay buffer and centrifuged at 20,000 *g* (15 min). The pellet was re-suspended in the same volume of cold buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 10 mM $MgCl_2$, 1.8 mM $CaCl_2$) and centrifuged twice as above with 30 min of incubation on ice in between the centrifugation steps. The resulting pellet was re-suspended, homogenized in assay buffer by using a glass/teflon homogenizer and assayed as described above for CHO cell membranes.

2.6. Bioluminescence resonance energy transfer (BRET) measurements

CHO and CHO-$GABA_B$ cells were transiently transfected with plasmids encoding Gαo-Rluc, Venus-Gγ2, Flag-Gβ2 and Myc-KCTD8, Myc-KCTD12 or Myc-KCTD16 and seeded into 96-well microplates (Greiner Bio-One). Cells were washed with PBS 24 h after transfection. BRET was measured in an Infinite® F500 microplate reader (Tecan) after injection of 5 μM Coelenterazine h (NanoLight Technologies). Baclofen was added at a final concentration of 10 μM or 100 μM. GS39783 was added at the final concentration of 10 μM, 5 min prior to measurement. Luminescence and fluorescence signals were detected sequentially with an integration time of 200 ms. The BRET ratio was calculated as the ratio of light emitted by Venus-Gγ2 (530–570 nm) over light emitted by Gαo-Rluc (370–470 nm) and corrected by subtracting ratios obtained with the Rluc fusion protein alone. The results were expressed in mBRET units determined as net BRET × 1000. Each data point was obtained using duplicate wells. The curves were fitted using GraphPad Prism 5.01 software ("Plateau followed by one-phase decay").

2.7. Electrophysiology

Kir3 currents were recorded at 30–32 °C in artificial cerebrospinal fluid containing 145 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 10 mM HEPES, pH 7.3. Patch pipettes were pulled from borosilicate glass capillaries (resistance of 3–5 MΩ) and filled with a solution containing 140 mM K-gluconate, 4 mM NaCl, 5 mM HEPES, 2 mM $MgCl_2$, 1.1 mM EGTA, 2 mM Na_2-ATP, 5 mM phosphocreatine, 0.6 mM Na_3-GTP, at pH 7.25 (adjusted with KOH). $GABA_B$R responses were evoked at −50 mV by fast application of 1–100 μM baclofen (Ascent Scientific) with a multiple channel perfusion valve control system (VC-8, 3 barrels, Warner Instruments). Data were acquired with a MultiClamp 700B (Molecular Devices), low-pass filtered at 2 kHz and digitized at 10 kHz using a Digidata 1440A interface (Molecular Devices) driven by pClamp 10.3 software. Whole-cell currents were analyzed using Clampfit 10.3 software (Molecular Devices). Data are expressed as mean ± S.E.M. and were analyzed with GraphPad Prism 5.01 software. EGFP-expressing CHO cells were identified via epifluorescence using an FITC filter set and patched under oblique illumination optics (BX51WI; Olympus).

2.8. Statistics

Curves for radioligand displacement and [^{35}S]GTPγS binding at different GABA concentrations were estimated by nonlinear regression. pKI, pEC_{50} ($-\log_{10}EC_{50}$) and E_{max} values represent the mean ± S.E.M of at least three independent experiments. Levels of statistical significance were set at *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$. Extra sums-of-squares F-tests were used for pKI and pEC_{50}, using the GraphPad Prism 5.01 software. Two-way ANOVA, followed by a post hoc Sidak's multiple comparison test was used for E_{max}. Kruskal–Wallis test followed by Dunn's multiple comparison test was used to determine basal [^{35}S]GTPγS binding and baseline BRET in the absence of agonist.

3. Results

3.1. Effects of the KCTDs on GABA binding-affinity at recombinant and native $GABA_B$Rs

In order to determine if the KCTDs allosterically influence agonist binding-affinity at $GABA_{B1}$, we transiently expressed $GABA_{B1b}$ or $GABA_{B(1b,2)}$ in the absence and presence of KCTD8, KCTD12 or KCTD16 in HEK293T cells. Approximately equal expression of ectopic $GABA_{B1b}$ and $GABA_{B2}$ in cells was verified using protein extracts from transiently transfected HEK293T cell membrane fractions (Suppl. Fig. 1). We used [^{3}H]CGP54626A, a competitive $GABA_B$R antagonist (Kaupmann et al., 1997), for radioligand displacement experiments with membranes of transfected cells. In agreement with earlier studies (Kaupmann et al., 1997, 1998), the affinity of GABA at $GABA_{B1b}$ was ~10-fold lower than at the $GABA_{B(1b,2)}$ heteromer and ~100-fold lower than at rat cortical membranes (Fig. 1A). Co-expression of the KCTDs slightly increased GABA affinity at $GABA_{B(1b,2)}$ (Fig. 1A and Table 1). This increase in affinity reached significance for KCTD8 and KCTD16 but not for KCTD12 (F-test). These experiments show that binding of KCTD8 or KCTD16 to $GABA_{B2}$ exerts a small positive allosteric effect on the GABA binding-site of $GABA_{B1}$. The GABA binding-affinity at all recombinant $GABA_B$R subtypes analyzed here was still significantly lower than the affinity at native receptors ($p < 0.001$ for rat cortical membranes vs. KCTD8, KCTD12 and KCTD16; F-test). It is conceivable that in transfected HEK293T cells distinct receptor populations contribute to the measured GABA binding-affinity. In line with this HEK293T cells transiently transfected with $GABA_{B1b}$ in combination with $GABA_{B2}$ alone or together with KCTD8, KCTD12, or KCTD16 show Hill coefficients of 0.68, 0.77, 0.66 and 0.72, respectively. In contrast HEK293T cells transiently transfected with $GABA_{B1b}$ alone or rat cortical membranes exhibit a Hill coefficient close to 1.0 indicative of a single binding site (Table 1). While it cannot be ruled out that the deviation of the Hill coefficients from 1 and the lower overall affinity of recombinant receptors compared to native receptors are caused by overexpression in HEK293T cells it is also possible that still unknown factors are responsible for the higher affinity of native receptors. The GABA binding-affinity of full brain membranes of wild-type (WT) and *Kctd8/12/16*$^{-/-}$ knock-out (3KO) mice (Turecek et al., 2014) are similar ($p = 0.503$; Fig. 1B and Table 2) and rule out a pronounced effect of the KCTDs on the overall binding affinity in the mouse brain. In summary, the data show that KCTD8 and KCTD16 exert a small allosteric effect on the orthosteric agonist binding-site in a recombinant expression system but do not significantly influence the overall agonist binding affinity in native membranes.

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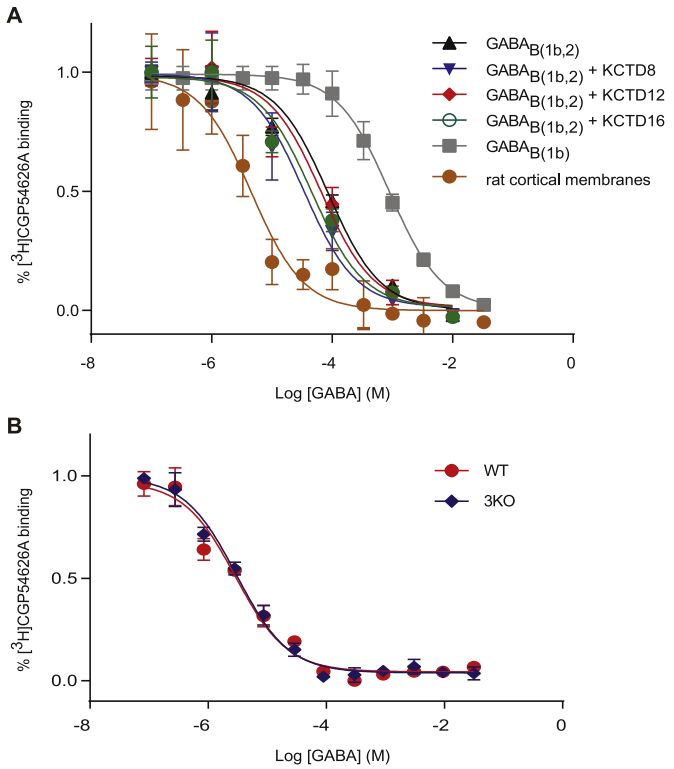

Fig. 1. Effects of the KCTDs on [^{3}H]CGP54626A displacement by GABA. (A) [^{3}H]CGP54626A displacement curves with membranes of HEK293T cells transiently transfected with either $GABA_{B(1b,2)}$ alone (black triangles, point up) or $GABA_{B(1b,2)}$ in combination with KCTD8 (blue triangles, point down), KCTD12 (red diamonds) or KCTD16 (open green circles). [^{3}H]CGP54626A displacement curves with membranes of HEK293T cells transfected with $GABA_{B1b}$ alone (gray squares) and with $GABA_BRs$ in rat cortical membranes (brown circles) are shown as controls. Displacement of [^{3}H]CGP54626A (5 nM) was performed with increasing concentrations of GABA. Data points represent means ± S.E.M. of 3–6 independent experiments, each performed in quadruplicates. A summary of relevant parameters is given in Table 1. (B) [^{3}H]CGP54626A displacement curves with brain membranes of wild-type (WT, red circles) or *Kctd8/12/16*$^{-/-}$ triple KO mice (3KO, blue diamonds). Displacement of [^{3}H]CGP54626A (5 nM) was performed with increasing concentrations of GABA. Data points represent means ± S.E.M. of 3 independent experiments, each performed in quadruplicates. A summary of relevant parameters is given in Table 2.

3.2. Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in [^{35}S]GTPγS binding experiments

To determine whether the KCTDs influence allosteric modulation of $GABA_BRs$ by a PAM we used GS39783 in combination with the [^{35}S]guanosine 5′-O-(3-thio)triphosphate ([^{35}S]GTPγS) binding assay, a well-established functional assay for $GABA_BRs$ (Urwyler et al., 2005, 2003). First, we analyzed G-protein activation in membranes of CHO-$GABA_B$ cells stably expressing $GABA_{B(1b,2)}$ in the absence or presence of KCTDs. For these experiments, we lentivirally transduced CHO-$GABA_B$ cells (Urwyler et al., 2001) to generate two additional cell lines stably expressing $GABA_{B(1b,2)}$ together with KCTD8 or KCTD12. KCTD12 represents KCTD12 and KCTD12b that both produce desensitizing Kir3 currents upon receptor activation. In contrast, KCTD8 represents KCTD8 and KCTD16 that produce largely non-desensitizing currents (Schwenk et al., 2010). [^{35}S]GTPγS binding assays were performed with membranes of all three cell lines, either in the absence or presence of 10 μM of GS39783 (Fig. 2A). Confirming earlier studies (Urwyler et al., 2005, 2003), we found that GS39783 significantly increased both potency and efficacy of GABA to stimulate [^{35}S]GTPγS binding in CHO-$GABA_B$ control cells (ø KCTD, Table 3). Similarly, GS39783 significantly increased both potency and efficacy of GABA at membranes of CHO-$GABA_B$ cells expressing KCTD8 or KCTD12 (Table 3, F-test for potency, two-way analysis of variance (ANOVA) with subsequent post-hoc Sidak's multiple comparison test for efficacy). Of note, the KCTDs did not significantly alter the potency of GABA at $GABA_BRs$ in the absence of GS39783 when compared to control CHO-$GABA_B$ cells (KCTD8: $p = 0.677$; KCTD12: $p = 0.114$). With GS39783-treated membranes the potency in the presence of KCTD8 but not KCTD12 was significantly but slightly decreased compared to control cells (KCTD8: $p = 0.003$; KCTD12: $p = 0.876$). This supports that KCTD8 exerts a weak allosteric influence on the PAM binding-site or the G-protein binding-site in the presence of the PAM. Furthermore, the GS39783-induced increase in the efficacy was significantly higher in the presence of KCTD8 than in control or KCTD12-expressing CHO-$GABA_B$ cells (Fig. 2A, $p < 0.001$ for KCTD8 vs. ø KCTD and KCTD8 vs. KCTD12), while there was no significant difference in the increase in efficacy between control and KCTD12-expressing cells ($p = 0.0608$ for ø KCTD vs. KCTD12).

The above data indicate that GS39783-modulation of the efficacy of $GABA_BR$ responses is remarkably large with KCTD8. One possible explanation for the larger increase in efficacy with KCTD8 is that KCTD8 reduces the efficacy in the absence of GS39783. A similar phenomenon was reported earlier for partial agonists that are more amenable to allosteric modulation of $GABA_BRs$ by PAMs than full agonists (Mannoury la Cour et al., 2008; Urwyler et al., 2005). In order to determine if KCTD8 reduces basal G-protein activation of $GABA_BRs$, we performed [^{35}S]GTPγS binding assays in the absence of agonist, but in the presence of a low GDP concentration (1 μM) (Roberts and Strange, 2005) to facilitate detection of agonist-independent receptor activity (Strange, 2010). Indeed, KCTD8 (Fig. 2B) led to a small but significant decrease in basal G-protein activation in the unstimulated state (86% of control CHO-$GABA_B$ cells, $p = 0.009$), while KCTD12 has no such effect (97% of control, $p = 0.654$). This indicates that KCTD8 decreases basal G-protein activation in the absence of agonist.

In summary, these experiments show that GS39783 increases agonist potency and efficacy both in the absence and presence of KCTDs in [^{35}S]GTPγS binding experiments. Together with the

Table 1
Effect of the KCTDs on [^{3}H]CGP54626A displacement by GABA. The binding of the orthosteric ligand [^{3}H]CGP54626A to recombinant $GABA_BRs$ on membranes of HEK293T cells and native $GABA_BRs$ on rat cortical membranes was measured as described in the Materials and Methods section. Displacement curves were constructed as illustrated in Fig. 1A. The results shown are means ± S.E.M. from (*N*) individual experiments.*, $p < 0.05$,**, $p < 0.01$,***, $p < 0.001$ compared to control $GABA_{B(1b,2)}$ receptors using an Extra sums-of-squares F-test.

$GABA_BR$	pKi GABA (−log M)	Hill coefficient	*N*
$GABA_{B(1b,2)}$	4.16 (±0.04)	0.68	6
$GABA_{B(1b,2)}$ + KCTD8	4.43 (±0.07)**	0.77	6
$GABA_{B(1b,2)}$ + KCTD12	4.27 (±0.04)	0.66	6
$GABA_{B(1b,2)}$ + KCTD16	4.31 (±0.06)*	0.72	6
$GABA_{B1b}$ homomer	3.07 (±0.03)***	1.11	3
Rat cortical membranes	5.23 (±0.09)***	0.91	4

Table 2
Effect of the KCTDs on [^{3}H]CGP54626A displacement from native $GABA_BRs$ by GABA. The binding of the orthosteric ligand [^{3}H]CGP54626A to native $GABA_BRs$ on brain membranes of wild-type (WT) or *Kctd8/12/16*$^{-/-}$ knockout (3KO) mice was measured as described in the Materials and Methods section. Displacement curves were constructed as illustrated in Fig. 1B. The results shown are means ± S.E.M. from (*N*) individual experiments. Results are compared to native $GABA_BRs$ of wild-type mice using an Extra sums-of-squares F-test.

$GABA_BR$	pKi GABA (−log M)	Hill coefficient	*N*
WT	5.58 (±0.10)	0.80	3
3KO	5.50 (±0.06)	0.86	3

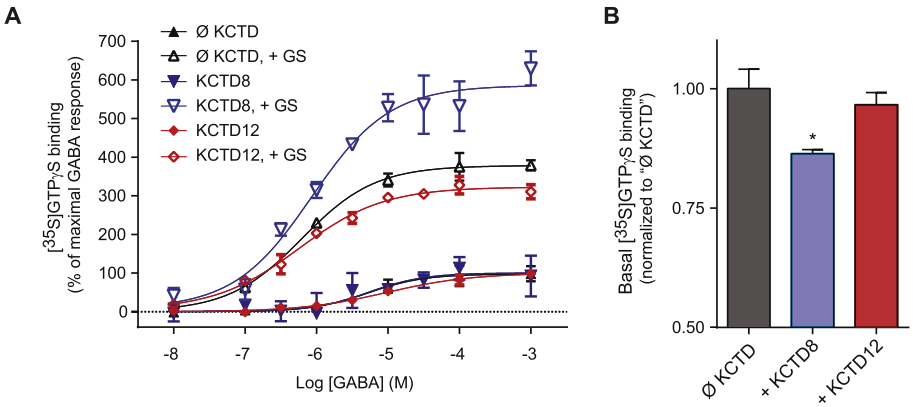

Fig. 2. Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in [^{35}S]GTPγS binding experiments. (A) GABA-induced [^{35}S]GTPγS binding to membranes of CHO-$GABA_B$ cells stably expressing either $GABA_{B(1b,2)}$ alone (ø KCTD, black triangles, point up) or $GABA_{B(1b,2)}$ in combination with KCTD8 (blue triangles, point down) or KCTD12 (red diamonds). Filled symbols indicate the absence, empty symbols the presence of GS39783 (10 μM, +GS). Data points represent the stimulation of [^{35}S]GTPγS binding by the indicated concentrations of GABA above basal binding (0%) normalized to the maximal GABA response in the absence of GS39783 (100%). Data points are means ± S.E.M. of three independent experiments, each performed in quadruplicates. A summary of relevant parameters is given in Table 3. (B) Summary bar graph of basal [^{35}S]GTPγS binding, performed in the presence of 1 μM of GDP, in CHO-$GABA_B$ cells expressing $GABA_{B(1b,2)}$ without (ø KCTD, black) or with KCTD8 (blue) or KCTD12 (red). One representative experiment out of three is shown. Results are means ± S.E.M. of quadruplicates. *, $p < 0.05$ of Kruskal–Wallis test followed by Dunn's multiple comparison test.

radioligand displacement experiments (Fig. 1), this supports that the KCTDs exert no or little allosteric influence on allosteric and orthosteric binding sites in principal receptor subunits. Of note, KCTD8 reduces the basal level of receptor activity. GS39783 can overcome reduced G-protein coupling in the presence of KCTD8 and therefore induces a significantly larger increase in the maximal receptor response.

3.3. Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in BRET G-protein activation experiments

The [^{35}S]GTPγS binding experiments showed that GS39783 acts as a PAM in a functional assay in the absence and presence of the KCTDs. The [^{35}S]GTPγS binding experiments additionally indicated that KCTD8 reduces basal G-protein activation by the receptor, but that increased activation can still be obtained in the presence of GS39783. To corroborate these findings, we monitored $GABA_BR$-induced G-protein activation in BRET experiments (Digby et al., 2006; Frank et al., 2005; Turecek et al., 2014). We transiently transfected CHO-$GABA_B$ cells with the donor fusion protein Gαo-Rluc, the acceptor fusion protein Venus-Gγ2, Gβ2 and each of the KCTDs. We then determined the magnitude of the BRET change between Gαo-Rluc and Venus-Gγ2 during G-protein activation induced by baclofen (10 μM and 100 μM) in the absence and presence of GS39783. Of note, baclofen concentrations lower than 10 μM did not lead to measurable changes in BRET.

GS39783 (10 μM) significantly increased the magnitude of the BRET change induced by baclofen in the absence and presence of KCTDs (Fig. 3A–C). Of note, we recently reported a significantly larger magnitude of the BRET change with KCTD12, which reflects binding of KCTD12 to an activity-dependent binding-site on the βγ subunits of the G-protein (Turecek et al., 2014). This KCTD12-specific conformational rearrangement of the G-protein is only seen with the high concentration of baclofen. Activity-dependent binding of KCTD12 to βγ requires unbinding of Gα from Gβγ (Turecek et al., 2014) and may therefore depend on substantial G-protein dissociation, offering a possible explanation for the requirement of a high baclofen concentration. The KCTD12-specific increase in the change of BRET is further increased by GS39783. This indicates that PAMs and KCTD12 affect the G-protein rearrangement independent of each other, with KCTD12 primarily acting at the G-protein (Turecek et al., 2014).

Interestingly, KCTD8, but not KCTD12 or KCTD16, showed a significantly decreased baseline BRET in the absence of baclofen, which reflects the basal G-protein conformation (Fig. 3B and C, right, also compare traces of KCTD8 with other traces in Fig. 3A). To determine if the decreased baseline BRET requires binding of KCTD8 to $GABA_BR$, BRET studies were performed with CHO cells that lacked $GABA_BRs$ or expressed adenosine receptor 1 (A_1). In these cells KCTD8 did not significantly decrease the baseline BRET levels when compared to controls ($p = 0.793$ for CHO cells without $GABA_BR$ ($n = 24$); $p = 0.252$ for CHO cells expressing A_1 ($n = 27$)). In summary, the BRET measurements support that GS39783 increases agonist-induced G-protein activation irrespective of the KCTDs. Moreover, KCTD8 reduces basal G-protein activation when associated with $GABA_BRs$.

Table 3
Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in [^{35}S]GTPγS binding experiments. Concentration response curves were measured (Fig. 2) from membranes of CHO-$GABA_B$ cells, stably expressing $GABA_BRs$ without or with KCTDs, both in the absence and presence of the positive allosteric modulator GS39783 (GS). Because the maximal effect of GABA alone differed between experiments, the data were normalized to the maximal effect (E_{max}) obtained with saturating concentrations of GABA alone in the absence of GS (100%) and basal activity without GABA (0%). The results shown are means ± S.E.M from (N) individual experiments.**, $p < 0.01$,***, $p < 0.001$ compared to non- GS-treated control values (Extra sums-of-squares F-test for pEC_{50} and two-way ANOVA followed by a post-hoc Sidak's multiple comparison test for E_{max}).

	pEC_{50} GABA (- log M)	E_{max}	N
ø KCTD	5.3 (±0.1)	97.5 (±7.0)	3
ø KCTD, +GS	6.2 (±0.0)***	378.4 (±7.3)***	
KCTD8	5.2 (±0.2)	100.5 (±15.8)	3
KCTD8, +GS	5.9 (±0.0)**	586.0 (±20.7)***	
KCTD12	5.1 (±0.1)	100.0 (±5.9)	3
KCTD12, +GS	6.2 (±0.0)***	322.8 (±7.0)***	

3.4. Effects of the KCTDs on allosteric modulation of native $GABA_BRs$ in [^{35}S]GTPγS binding experiments

[^{35}S]GTPγS binding and BRET studies show that GS39783 acts as a PAM at recombinant $GABA_BRs$ assembled with KCTDs. We further investigated whether GS39783 exerts PAM activity at native

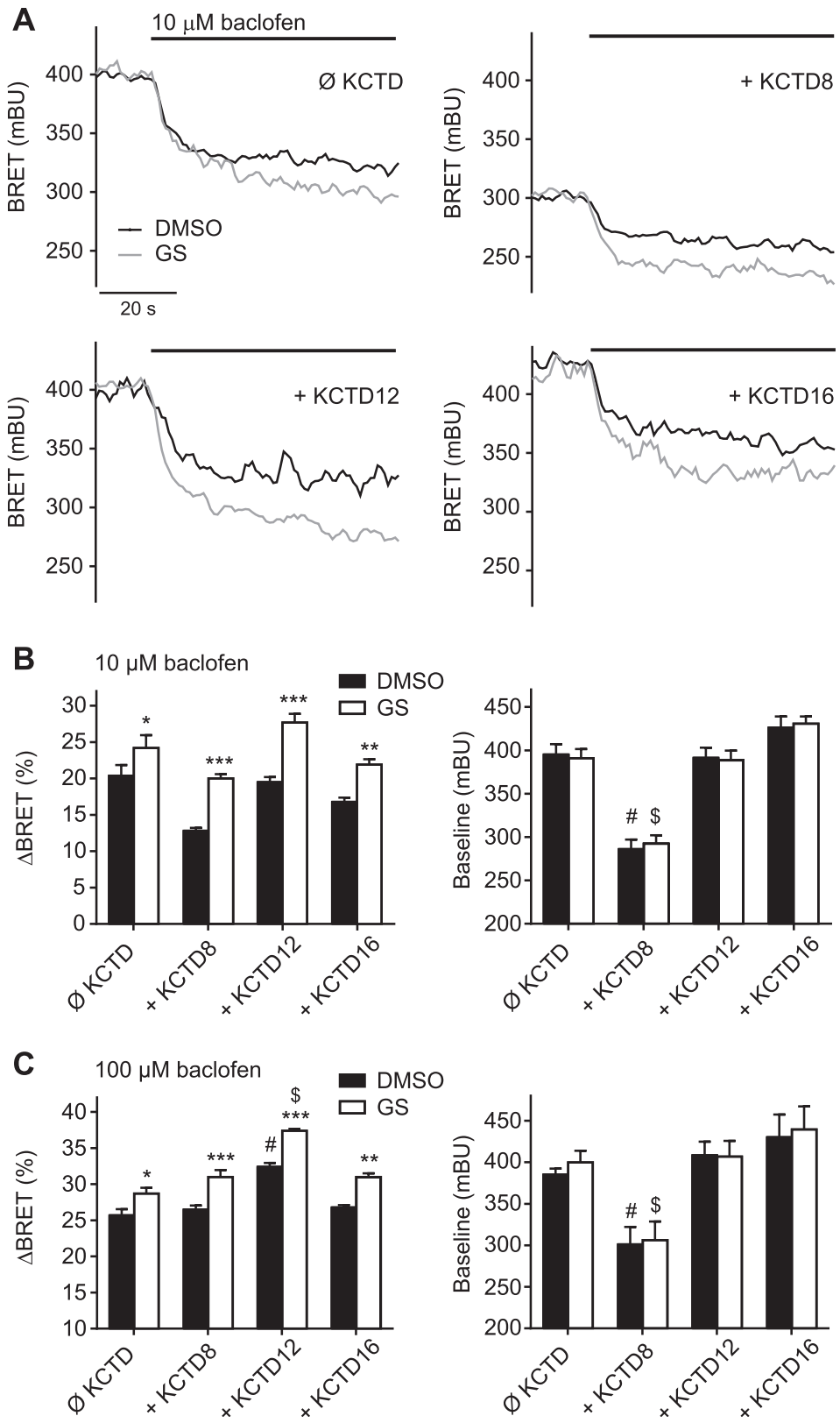

Fig. 3. Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in BRET G-protein activation assays. (A) Representative baclofen-induced changes in the BRET signal (milli BRET units, mBU) with CHO-$GABA_B$ cells co-expressing Gαo-Rluc, Venus-Gγ2 and Gβ2 with or without KCTD8, KCTD12 or KCTD16 in the absence (black) or presence (gray) of GS39783 (GS, 10 μM, 5 min pre-incubation). (B) Summary bar graph of BRET changes upon 10 μM baclofen application (changes in BRET (ΔBRET) in % of basal BRET, left) and baseline BRET (in mBU, right). Data are means ± S.E.M. of eight individual experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared to the respective DMSO treatment. #, $p < 0.001$; compared to all DMSO treatments. $, $p < 0.001$; compared to all GS treatments. (C) Summary bar graph of BRET changes upon 100 μM baclofen application (left) and baseline BRET (right). Data are means ± S.E.M. of 4–7 individual experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared to the respective DMSO treatment. #, $p < 0.05$; compared to all DMSO treatments. $, $p < 0.01$; compared to all GS treatments.

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$GABA_BRs$ in the absence of KCTDs, using the [^{35}S]GTPγS binding assay and whole brain membranes of 3KO mice. Of note, KCTD12b is only expressed in the medial habenula and does not substantially influence biochemical assays in whole brain membranes (Metz et al., 2011; Schwenk et al., 2010). As expected, GS39783 increased both potency and efficacy of GABA at $GABA_BRs$ of wild-type (WT) mice (Perdona et al., 2011; Urwyler et al., 2003) (Fig. 4). The lack of KCTDs in 3KO mice did not influence the potency of GABA in the absence ($p = 0.822$) or presence of GS39783 ($p = 0.464$). Furthermore, the lack of KCTDs did not influence the ability of GS39783 to increase the efficacy of GABA in 3KO membranes ($p > 0.999$) (Table 4). In summary, GS39783 had similar PAM activity at native $GABA_BRs$ irrespective of whether KCTDs were present or not.

3.5. Effects of the KCTDs on allosteric modulation of $GABA_BR$-activated Kir3 currents in CHO cells

We next analyzed whether GS39783 allosterically modulates $GABA_BR$-activated Gβγ signaling in the presence or absence of KCTDs. Kir3 currents were evoked every 5 min by a 40 s application of baclofen (1 μM, ~EC_{20}, Fig. 5A). This was first done in the absence (−GS) and then in the presence of GS39783 (+GS, 10 μM, bath application for 10 min). GS39783 had no effect on holding currents (Suppl. Fig. 2A) but led to an approximately two-fold increase of the Kir3 current peak amplitude both in the absence and presence of KCTDs (Fig. 5A and B). GS39783 accentuated kinetic effects of KCTD12 on $GABA_BR$ signaling described earlier (Schwenk et al., 2010; Turecek et al., 2014). Specifically, GS38793 shortened the rise-time and increased the relative desensitization of evoked Kir3 currents (Fig 5B). The effects of KCTD12 on rise-time and desensitization are dependent on the baclofen concentration (Suppl. Fig. 2B and C). It is therefore likely that GS39783 shortens the rise-time and increases desensitization in the presence of KCTD12 by increasing agonist binding-affinity at the receptor. Interestingly, GS39783 did not significantly increase the plateau current during prolonged KCTD12-induced desensitization (+GS39783: 19.7 ± 6.2 pA,−GS39783: 13.1 ± 6.0 pA, $p = 0.055$, Fig. 5A). Since KCTD12 induces desensitization by interfering with

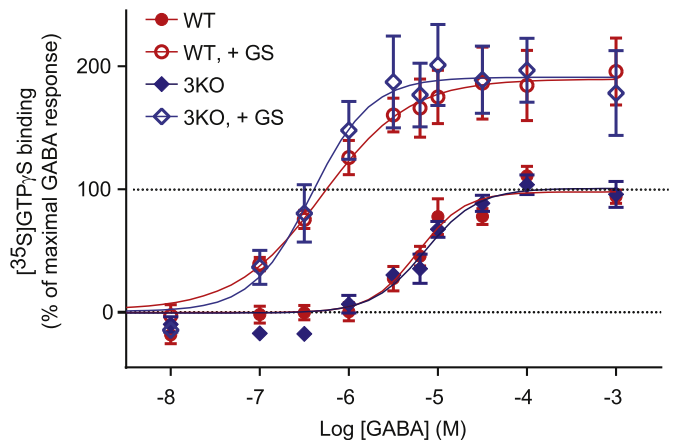

Fig. 4. Effects of the KCTDs on allosteric modulation of native $GABA_BRs$ in [^{35}S]GTPγS binding experiments. Effect of GS39783 on GABA-induced [^{35}S]GTPγS binding to brain membranes of wild-type (WT, red circles) or *Kctd8/12/16*$^{-/-}$ triple knockout mice (3KO, blue diamonds). Filled symbols indicate the absence, empty symbols the presence of GS39783 (10 μM, +GS). Data points represent the stimulation at different GABA concentrations above basal binding (0%) normalized to the maximal GABA response in the absence of GS39783 (100%). Data points are means ± S.E.M. of four independent experiments, each performed in quadruplicates. A summary of relevant parameters is given in Table 4.

Table 4
Effects of the KCTDs on allosteric modulation of native $GABA_BRs$ in [^{35}S]GTPγS binding experiments. Concentration response curves were measured (Fig. 4) from brain membranes of wild-type (WT) or *Kctd8/12/16*$^{-/-}$ knockout (3KO) mice, both in the absence and presence of the positive allosteric modulator GS39783 (GS). Because the maximal effect of GABA alone differed between experiments, the data were normalized to the maximal effect (E_{max}) obtained with saturating concentrations of GABA alone in the absence of GS (100%) and basal activity without GABA (0%). The results shown are means ± S.E.M from (*N*) individual experiments.*, $p < 0.05$,***, $p < 0.001$ compared to non-GS-treated control values (Extra sums-of-squares F-test for pEC_{50} and two-way ANOVA followed by a post-hoc Sidak's multiple comparison test for E_{max}).

	pEC_{50} GABA (−log M)	E_{max}	*N*
WT	5.4 (±0.1)	100.0 (±0.6)	4
WT, +GS	6.3 (±0.1)***	196.3 (±27.8)*	
3KO	5.3 (±0.1)	100.0 (±0.1)	4
3KO, +GS	6.4 (±0.1)***	193.5 (±28.8)*	

Gβγ activation of the Kir3 channel (Turecek et al., 2014) the PAM activity at the receptor may no longer be very effective during maximal KCTD12-mediated inhibition of Gβγ signaling. Altogether the data are consistent with the PAM potentiating KCTD12-mediated effects at the G-protein by increasing agonist affinity at the receptor and hence receptor activation at a given concentration of agonist.

3.6. Effects of KCTDs on allosteric modulation of $GABA_BR$-activated K^+ currents in hippocampal neurons

KCTD8, 12, 12b and 16 are differentially expressed in the brain (Metz et al., 2011; Schwenk et al., 2010). KCTD12 and 16 expression is high in the hippocampus while KCTD8 expression is low in this area. KCTD12b is exclusively expressed in the medial habenula. Using cultured hippocampal neurons from 3KO mice and control WT mice we analyzed whether GS39783 has similar PAM activity in the absence and presence of KCTD proteins.

Native K^+ currents exhibit strong run-down after repeated baclofen application. We therefore studied the modulatory effect of GS39783 during steady-state application of baclofen. We applied a low concentration of baclofen (1 μM) for 1 min and then co-applied GS39783 (10 μM) for an additional minute (Fig 6). Baclofen evoked K^+ currents exhibited little desensitization. GS39783 increased the K^+-current amplitude both in WT and 3KO neurons. This increase in amplitude was not significantly different between 3KO (27 ± 5%) and WT (40 ± 9%) neurons ($p = 0.40$). This result confirms that GS39783 is equally effective as a PAM at native $GABA_BRs$ with or without KCTDs.

4. Discussion

It is well established that the $GABA_{B1}$ and $GABA_{B2}$ subunits of $GABA_BRs$ are allosterically coupled through multiple interactions in their extracellular and transmembrane domains (Monnier et al., 2011). Likewise, $GABA_BRs$ undergo allosteric interactions with the G-protein (Hill et al., 1984; Pin et al., 2004). Constitutive association of $GABA_BRs$ with auxiliary KCTD subunits not only generates molecularly distinct receptor subtypes but also introduces the KCTDs as novel factors that potentially allosterically regulate the ternary receptor complex (Gassmann and Bettler, 2012; Schwenk et al., 2010). The KCTDs bind to both receptor and G-protein, which influences the kinetics of the receptor response through stabilizing the G-protein at the receptor and through direct effects on G-protein signaling (Turecek et al., 2014). In principle, the KCTDs could allosterically regulate the receptor through direct effects at $GABA_{B2}$ and/or through indirect effects at the receptor-associated

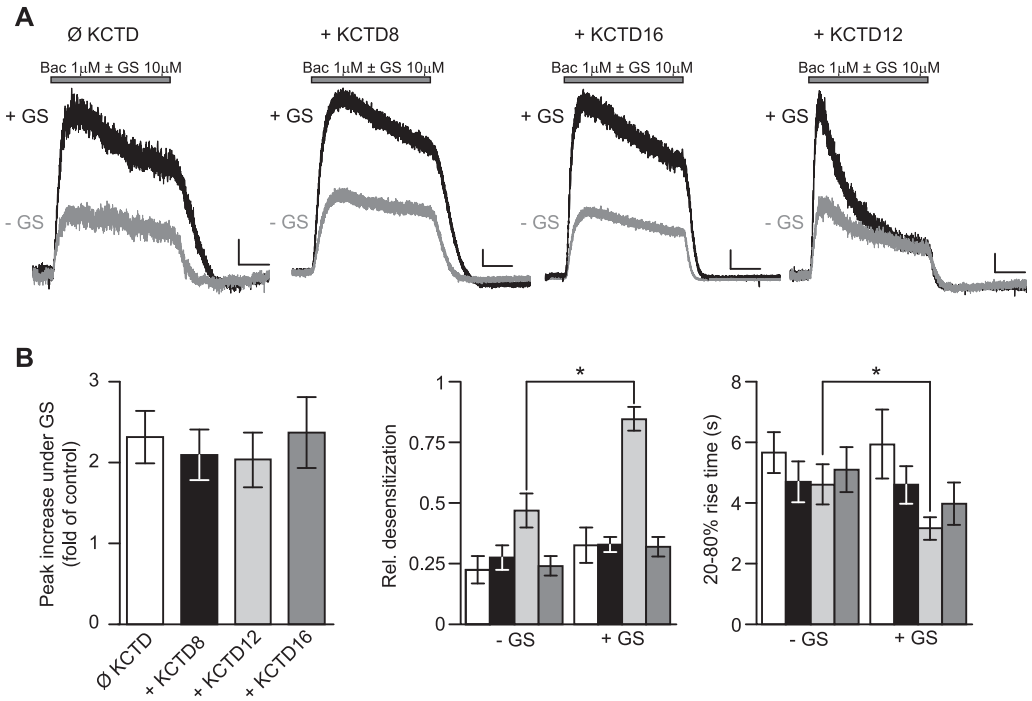

Fig. 5. Effects of the KCTDs on allosteric modulation of $GABA_BR$-activated Kir3 currents in transfected CHO cells. (A) Representative traces of $GABA_B$-activated Kir3 currents recorded at −50 mV from CHO-$GABA_B$ cells co-expressing Kir3 channels with or without KCTD proteins. Control traces evoked by 1 μM baclofen (Bac) are shown in gray (- GS), traces evoked by co-application of 1 μM baclofen + 10 μM of GS39783 in black (+GS). Pre-incubation with GS39783 by bath was for 10 min. Scaling: 10 pA/10 s. (B) Summary bar graphs showing the effect of GS39783 on peak amplitude, relative desensitization and rise-time of Kir3 currents. ø KCTD, white; +KCTD8, black; +KCTD12, light gray; +KCTD16, dark gray. Data are expressed as mean ± S.E.M.; *, $p < 0.05$, Mann–Whitney test on paired data.

G-protein. The KCTDs could also influence the activity of PAMs that, like the KCTDs, bind to $GABA_{B2}$. PAMs at $GABA_BRs$ increase both agonist binding-affinity and efficacy of the receptor response (May et al., 2007; Urwyler et al., 2003), showing that PAMs modulate both the orthosteric agonist binding-site and G-protein activation. PAMs may therefore also influence interactions between the KCTDs and the G-protein. In our studies we have addressed whether the KCTDs allosterically influence (i) the orthosteric agonist binding-site, (ii) G-protein activation and (iii) modulation by GS39783. Our data indicate that the KCTDs exert *per se* little allosteric influence on the orthosteric agonist binding-site or on G-protein activation. This shows that the increase in GABA potency observed in the presence of KCTDs with receptor-activated Kir3 currents (Schwenk et al., 2010) is not due to an increase in agonist binding-affinity but relates to KCTD effects on the G-protein cycle. The effects on the G-protein cycle are lost in the [^{35}S]GTPγS binding experiments, in which the G-protein cycle is interrupted. Allosteric regulation of the receptor complex by GS39783 is qualitatively similar in the absence and presence of the KCTDs. This further supports that the KCTDs primarily regulate the receptor response at the G-protein (Turecek et al., 2014). Differential regulation of the G-protein by the KCTDs, however, causes significant quantitative differences in the modulatory effect of GS39783 on the efficacy of the receptor response (KCTD8) or the increase in the relative desensitization of receptor-activated K^+-currents (KCTD12) in transfected cells. However, we did not observe significant differences between the modulatory effect of GS39783 on native receptors in WT and 3KO mice. This is likely due to the fact that native $GABA_BRs$ are heterogeneous. Whereas the $GABA_{B1}$ and $GABA_{B2}$ protomers are expressed by almost all neurons and glial cells (Gassmann and Bettler, 2012), the repertoire and abundance of the KCTDs varies among brain areas, neuronal populations and subcellular sites (Metz et al., 2011; Schwenk et al., 2010). Moreover, a significant fraction of native $GABA_BRs$ in the brain is devoid of any KCTDs (Turecek et al., 2014). Thus, it is likely that native $GABA_BRs$ assembled with specific KCTDs are also differentially modulated by GS39783, similar to the $GABA_BRs$ reconstituted with KCTDs in transfected cells, but that the responses of individual receptor populations are masked in the overall response of different receptor populations.

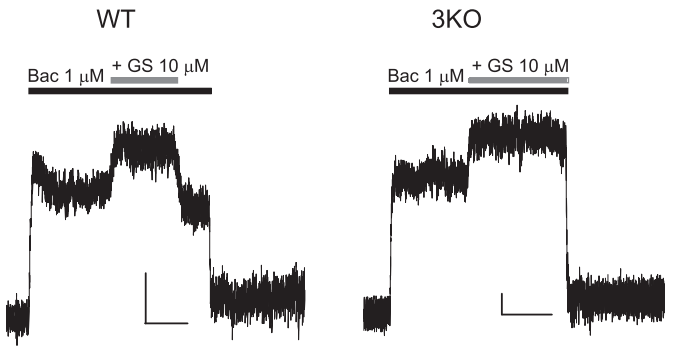

Fig. 6. Effects of the KCTDs on allosteric modulation of $GABA_BR$-activated K^+ currents in native neurons. Representative traces of $GABA_B$-activated Kir3 currents recorded at −50 mV from cultured hippocampal neurons of wild-type (WT, left) or *Kctd8/12/16*$^{-/-}$ triple knockout mice (3KO, right). 1 μM of baclofen (Bac, black bars) was applied for 90 s, then 10 μM of GS39783 (+GS, gray bars) was co-applied for an additional 90 s. Scaling: 10 pA/1 min.

The agonist binding experiments show that the KCTDs fail to increase agonist binding-affinity at recombinant $GABA_BRs$ to the level of native receptors. This suggests that other factors are responsible for the higher agonist binding-affinity of native $GABA_BRs$. Candidate factors are additional binding partners of $GABA_BRs$ (Schwenk et al., 2010) or posttranslational modifications

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of receptor components, such as for example phosphorylation of the $GABA_{B1}$ and $GABA_{B2}$ subunits (Couve et al., 2002; Guetg et al., 2010; Kuramoto et al., 2007).

KCTD8 reduces basal G-protein activation in the [^{35}S]GTPγS binding assay. Reduced basal G-protein activation can to some extent be overcome with GS39783, which induces a larger increase in efficacy with KCTD8 than with the other KCTDs. A similar phenomenon has been observed with partial agonists that are more amenable to allosteric modulation by PAMs than full agonists. This was for example shown with $GABA_B$Rs (Mannoury la Cour et al., 2008; Urwyler et al., 2005) and mGlu2 receptors (Schaffhauser et al., 2003). This supports that a less efficacious G-protein coupling – either due to the effects of a partial agonist or the presence of KCTD8 – allows for a bigger total increase in efficacy in the presence of the PAM. Consistent with a unique effect of KCTD8 on basal G-protein activation we selectively observed a change in the basal BRET between G-protein subunits in the combined presence of KCTD8 and $GABA_B$Rs. KCTD8, when associated with $GABA_B$Rs, therefore likely induces a conformational change in the G-protein that reduces basal G-protein activation by the receptor. Of note, the BRET experiments allow for the first time to distinguish KCTD8 and KCTD16 in a functional assay system.

Acknowledgments

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Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.08.020.

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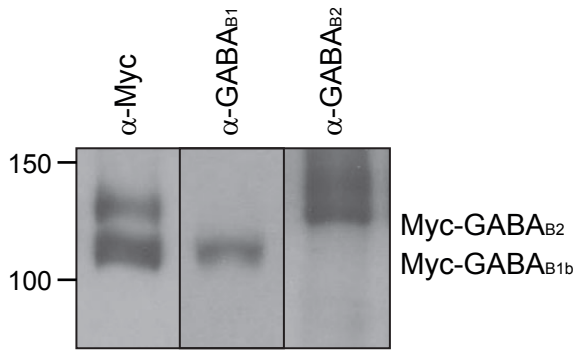
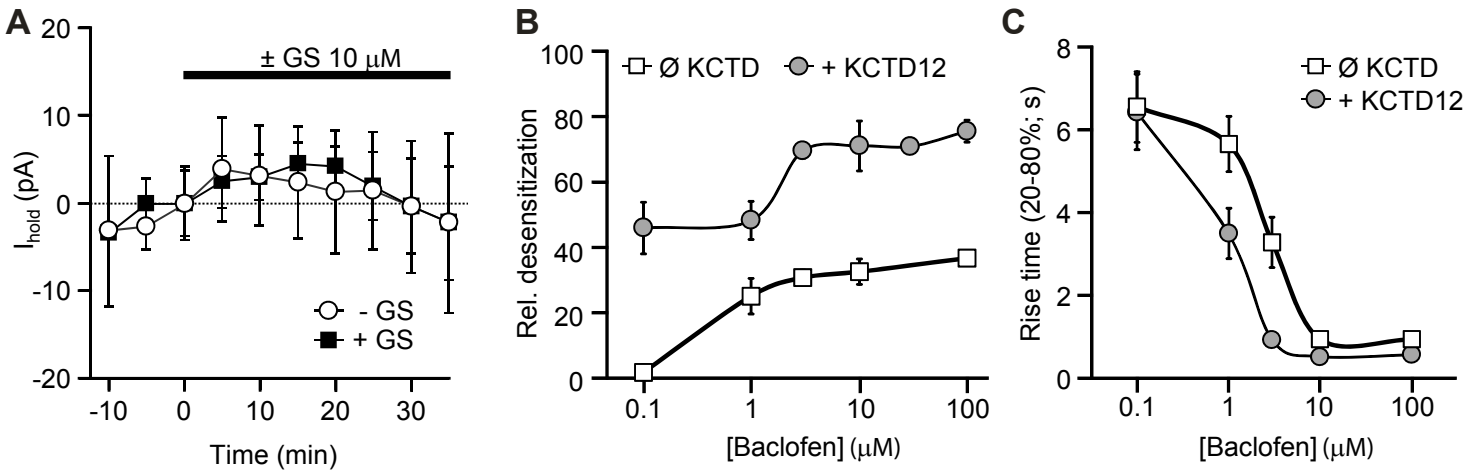


Supplementary Figure 1



Supplementary Figure 2

2.4 $GABA_B$ receptor signaling is controlled by complex formation of KCTD16, cullin3 and 14-3-3 proteins

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in preparation

Personal contribution

Design and realization of all experiments

Data analysis

Design of figures

Preparation of the manuscript

$GABA_B$ receptor signaling is controlled by complex formation of KCTD16, cullin3 and 14-3-3 proteins

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ABSTRACT

$GABA_B$ receptors are the G-protein coupled receptors (GPCRs) for GABA, the main inhibitory neurotransmitter in the central nervous system. As they are crucial for numerous neuronal processes, the activity of $GABA_B$ receptors needs to be temporally precise. The principal $GABA_B$ receptor subunits associate with auxiliary subunits, the K^+ channel tetramerization domain-containing proteins KCTD8, 12 and 16. In this study, we found that KCTD16, but not KCTD8 or 12, binds to 14-3-3 proteins. The binding is mediated by the C-terminal H2-domain of KCTD16 and regulates the expression of KCTD16 in a 14-3-3 isoform-specific manner. 14-3-3 ε promotes KCTD16 stability and tetramer formation whereas 14-3-3 γ promotes lysosomal degradation of KCTD16. Additionally, the interaction to KCTD16 enables 14-3-3 proteins to modulate the expression of the $GABA_{B2}$ subunit. Using a protein-fragment complementation assay, we found that the Gα-signaling of $GABA_B$ receptors is changed as a consequence of KCTD16 and 14-3-3 co-expression. We further identified cullin3 as a new interaction partner of KCTD16 and show that this interaction is affected by 14-3-3 proteins. We propose that $GABA_B$ receptors are substrates of the KCTD16-cullin3 complex. This provides a mechanism of how cell surface $GABA_B$ receptors can be targeted for lysosomal degradation, which is regulated by 14-3-3 proteins.

INTRODUCTION

Functional $GABA_B$ receptors are heterodimers that consist of $GABA_{B1}$ and $GABA_{B2}$ subunits. Whereas GABA binds to the $GABA_{B1}$ subunit, surface-targeting and receptor signaling is mediated by the $GABA_{B2}$ subunit (Couve et al., 2000, Calver et al., 2001, Bettler et al., 2004, Pin et al., 2004). $GABA_B$ receptors couple to $G\alpha_{i/o}$-type G-proteins and modulate the activity of adenylyl cyclase, G protein-gated inwardly rectifying K^+ channels and voltage-gated Ca^{2+} channels (Luscher and Slesinger, 2010, Chalifoux and Carter, 2011, Gassmann and Bettler, 2012). As $GABA_B$ receptor activity is fundamental for numerous neuronal processes, like synaptic plasticity or neuronal firing, its activity has to be precisely controlled. Receptor signaling is regulated at different time points of receptor maturation including the export from the endoplasmic reticulum, receptor desensitization, endocytosis and degradation (Benke et al., 2012). For functional $GABA_B$ receptors localized in the cell membrane, posttranslational modifications have a major impact on receptor signaling. Phosphorylation of $GABA_B$ receptors controls receptor stability, induces endocytosis, and differentiates between receptor recycling and degradation (Couve et al., 2002, Fairfax et al., 2004, Guetg et al., 2010, Maier et al., 2010, Terunuma et al., 2010, Adelfinger et al., 2014). Additionally, the binding of other proteins to $GABA_B$ receptors can contribute to the extent of receptor activity. The auxiliary receptor subunits

KCTD12 and 12b, for instance, induce fast desensitization of $GABA_B$-activated K^+ currents, whereas KCTD8 decreases the basal G-protein activation of $GABA_B$ receptors (Schwenk et al., 2010, Rajalu et al., 2014). Further, members of the 14-3-3 protein family were found to be associated with $GABA_B$ receptors and to modulate $GABA_B$ receptor signaling. Although 14-3-3 proteins are dispensable for surface trafficking of $GABA_B$ receptors, they are capable of dissociating the $GABA_{B1/2}$ receptor heterodimer and thereby impairing its signaling (Couve et al., 2001, Brock et al., 2005, Laffray et al., 2012).

In this study, we show that the interaction between KCTD16 and distinct members of the 14-3-3 family leads to changes in KCTD16 and $GABA_{B2}$ subunit expression. 14-3-3 γ binding promotes lysosomal degradation of KCTD16, whereas 14-3-3 ε binding stabilizes KCTD16 by increasing the affinity for KCTD16-tetramer formation. Moreover, we found that the interaction of KCTD16 with 14-3-3 proteins regulates the Gα-signaling of $GABA_B$ receptors. Using a protein-fragment complementation assay, we show that KCTD16 together with 14-3-3 ε increases $GABA_B$-mediated PKA inhibition, whereas KCTD16 together with 14-3-3 γ leads to a disinhibition. Together, we propose that KCTD16 is a cullin3 substrate adaptor that controls the amount of cell surface $GABA_B$ receptors, which in turn is regulated in a 14-3-3 isoform-dependent manner.

MATERIAL AND METHODS

Generation of expression plasmids - All KCTD cDNAs contained an N-terminal tag of three c-Myc epitopes and were cloned as described earlier (Seddik et al., 2012). To generate KCTD12-16H2Δ39 (12-16H2Δ39) and KCTD16Δ39 (16H2Δ39), a stop codon was inserted in KCTD12-16H2 and KCTD16 after residue Leu388. To generate KCTD16Δ60 (16H2Δ60), a stop codon was inserted in KCTD16 after residue Thr367. To generate KCTD12-16H2ΔN (12-16H2ΔN) and KCTD16ΔPEST (16ΔPEST), residues Pro280 to Cys314 in KCTD12-16H2 and residues Gly300 to Thr333 in KCTD16 were excised, respectively. The constructs KCTD12-16H2 (12-16H2), KCTD16ΔH2 (16ΔH2), KCTD16H2 (16H2) and KCTD12-16H2Δ60 (12-16H2Δ60) were described earlier (Seddik et al., 2012). The plasmids HA-14-3-3 γ and HA-14-3-3 ε were purchased from Addgene (Cambridge, MA, US). The plasmids RlucF1-RegPKA, RlucF2-CatPKA and RlucF2-RegPKA were a kind gift of E. Stefan (Montreal, Canada).

Cell culture - COS-1 cells were maintained in DMEM (Life Technologies, CA, US) supplemented with 10% FBS (GE Healthcare, Buckinghamshire, UK) at 37°C with 5% CO_2. CHO-K1 cells stably expressing $GABA_{B1}$ and $GABA_{B2}$ subunits were cultured as described (Urwyler et al., 2001). Lipofectamine 2000 (Life Technologies) was used for transient transfections. The amount of DNA in transfections was kept constant by supplementing with pCI plasmid DNA (Promega, WI, US).

Co-immunoprecipitation, Western blot analysis and Native PAGE - For co-immunoprecipitations, COS-1 cells were harvested, washed once with ice-cold PBS, and subsequently lysed in NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris/HCl, pH 7.4) supplemented with complete EDTA-free protease inhibitor mixture (Hoffmann-La Roche, Basel, Switzerland). After rotation for 10 min at 4°C, the lysates were cleared by centrifugation at 1000 x g for 10 min at 4°C. Lysates were directly used for Western blot analysis or immunoprecipitated. Lysates and immunoprecipitates were resolved using standard SDS-PAGE. All antibodies used for Western blot and immunoprecipitations are listed in Suppl. Table 1.

To study KCTD16 degradation, COS-1 cells were incubated with the following compounds for 18 h: Chloroquine diphosphate (100 µM, 4109, Tocris), Epoxomicine (2 µM, 324801, Millipore), MG132 (10 µM, M7449, Sigma Aldrich).

Native PAGE experiments were carried out as instructed by the manufacturer (Bio-Rad, CA, US). Briefly, cell lysates were prepared as for SDS-PAGE. Non-reducing sample buffer was composed of: 62.5 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 0.01% (w/v) bromophenol blue. The running buffer was composed of: 2.5 mM Tris, 19.2 mM glycine. 4-15% Mini-PROTEAN® TGX™ Gels and a HMW Native Marker Kit was purchased from Bio-Rad (456-1083S and 17-0445-01).

BRET measurements - BRET measurements were described earlier (Ivankova et al., 2013, Adelfinger et al., 2014). Briefly, COS-1 cells were transiently transfected with plasmids encoding Rluc-KCTD16/YFP-KCTD16 or Rluc-KCTD12/YFP-KCTD12 with or without HA-14-3-3 ε. Six hours after transfection, cells were distributed into 96-well microplates (Greiner Bio-One, Kremsmünster, Austria) at a density of 50,000 cells/well. Twenty-four hours after transfection, cells were washed and incubated in PBS containing 5 µM coelenterazine H (NanoLight Technologies, AZ, US) for 20 min at RT. Luminescence and fluorescence signals were detected sequentially using an Infinite® F500 Microplate Reader (Tecan, Männedorf, Switzerland). Each data point represents a technical quadruplicate.

Protein-fragment complementation assay - The PCA assay was performed as described earlier (Stefan et al., 2007). CHO-K1 cells stably expressing $GABA_{B1}$ and $GABA_{B2}$ subunits grown in 12-well plates were transfected with RlucF1-RegPKA and RlucF2-CatPKA (or RlucF2-RegPKA), myc-KCTD16 and HA-14-3-3 ε or γ. Six hours after transfection, cells were distributed into 96-well microplates. Fourty-eight hours later, cells were washed and incubated in PBS containing 10 µM forskolin and 5 µM coelenterazine H (NanoLight Technologies, AZ, US) for 20 min at RT. The increase in bioluminescence following baclofen injection (100 µM; 0417, Tocris Bioscience, UK) was recorded on an Infinite® F500 Microplate Reader (Tecan) with an integration time of 1 s. The curves were normalized to the point of injection and to the non-activatable control (RlucF1-RegPKA + RlucF2-RegPKA).

Data analysis – All data are presented as mean ± *SEM*. Statistical significance was assessed with the unpaired Student's *t* test using Prism 5.04 software (GraphPad, CA, US). BRET data were analyzed and fitted using a "one-site specific binding" equation (GraphPad).

RESULTS

KCTD16 associates with 14-3-3 proteins through its C-terminal H2-domain

14-3-3 proteins are known binding partners of the $GABA_B$ receptor (Couve et al., 2001, Brock et al., 2005, Laffray et al., 2012). Here, we observed that other members of the 14-3-3 family interact with members of the KCTD protein family, which are auxiliary subunits of the $GABA_B$ receptor. A schematic illustration of the three auxiliary subunits of the $GABA_B$ receptor is shown in Fig. 1A. We found that KCTD16 but not KCTD12 associates with 14-3-3 proteins (Fig. 1B). It is interesting to note that 14-3-3 ε and γ were identified as KCTD16 interacting proteins but not 14-3-3 ζ (data not shown), which was shown to bind the $GABA_{B1}$ subunit. As KCTD16 contains the H2-domain which is absent in KCTD12, it is conceivable that 14-3-3 proteins bind to this domain. To test this hypothesis, we used a chimeric protein, in which the H2-domain of KCTD16 was fused to the C-terminus of KCTD12 (12-16H2), and a deletion mutant, in which the H2-domain was removed from KCTD16 (16ΔH2). As predicted, both 14-3-3 ε and γ bound 12-16H2 but not 16ΔH2 (Fig. 1B). Moreover, the H2-domain itself (16H2) was

sufficient to bind the 14-3-3 proteins although the binding efficiency was strongly reduced (Fig. 1C). To investigate whether 14-3-3 proteins interact in general with H2-domains, we tested KCTD8, another H2 domain-containing KCTD protein, for interaction with 14-3-3 ε and γ. In contrast to KCTD16, KCTD8 was not able to associate with both 14-3-3 ε and γ (Fig. 1D). Together, these data show that KCTD16, but not KCTD8 or 12, interacts with 14-3-3 ε and γ through its C-terminal H2-domain.

In order to map the minimal binding site of KCTD16 for 14-3-3 proteins, we co-expressed truncations of the chimeric 12-16H2 protein in COS-1 cells for co-immunoprecipitation studies. Whereas the deletion of the N-terminal part of the H2-domain of 12-16H2 (deletion of amino acid residues 328-362; 12-16H2ΔN; Fig. 2A) did not affect the interaction with 14-3-3 ε or γ (Fig. 2B), deletions of the C-terminal part of the H2-domain of 12-16H2 affected the binding properties to 14-3-3 proteins (Fig. 2B and C). It was of interest to see that the deletion of the last 60 amino acid residues in 12-16H2Δ60 abolished the binding to both 14-3-3 isoforms, whereas the deletion of the last 39 amino acid residues in 12-16H2Δ39 interfered with the binding to 14-3-3 ε but not to 14-3-3 γ. As 14-3-3 proteins mostly bind to phosphorylated serines/threonins, we mutated selected serines/threonins, which are present within the C-terminal part of the H2-domain of KCTD16 but not in the H2-domain of KCTD8. However, the mutation of T369, S375 or S379 to alanine did not prevent the binding of 14-3-3 proteins (data not shown). These data indicate that 14-3-3 binding to KCTD16 is either phosphorylation-independent or that 14-3-3 binding is ensured by more than one binding site.

14-3-3 binding regulates KCTD16 protein expression

To assess the functional relevance of such interaction, we measured the expression levels of KCTD16 in the presence of 14-3-3 proteins. We observed that 14-3-3 protein co-expression affected the expression levels of the interaction partner KCTD16. Whereas 14-3-3 ε increased the protein levels of KCTD16, 14-3-3 γ decreased the expression of KCTD16 (Fig. 3A). These effects were dependent on direct interaction since both 14-3-3 isoforms did not affect protein levels of KCTD12 (Fig. 3B). Furthermore, the protein levels of KCTD16 truncation mutants were changed only if they were able to bind 14-3-3 proteins. The expression of the KCTD16 truncation mutant 16H2Δ39, which lacks the last 39 amino acid residues of the H2-domain equivalent to 12-16H2Δ39 and binds 14-3-3 γ but not 14-3-3 ε, is regulated exclusively by 14-3-3 γ (Fig. 3C). On the other hand, the expression level of the KCTD16 truncation mutant 16H2Δ60, which lacks the last 60 amino acid residues of the H2-domain equivalent to 12-16H2Δ60 and binds neither 14-3-3 isoforms, was unaffected by 14-3-3 co-expression (Fig. 3D). Together, these data show that direct binding is obligatory for 14-3-3 ε and γ to regulate the expression levels of KCTD proteins.

14-3-3 γ shifts the degradation of KCTD16 from the proteasome to the lysosome

14-3-3 proteins are well known to regulate the degradation of their binding partners (LeBron et al., 2006, Kasahara et al., 2010). Therefore, we investigated whether decreased protein levels of KCTD16 in the presence of 14-3-3 γ correspond to increased degradation of KCTD16. To test this hypothesis, we incubated COS-1 cells expressing either KCTD16 alone or together with 14-3-3 γ with inhibitors of the lysosome, chloroquine (CHQ), or proteasome, epoxomicine (EPO) or MG132 (MG). In cells co-expressing KCTD16 and 14-3-3 γ we observed a down-regulation of KCTD16 to ~55% which was inhibited in the presence of lysosome inhibitor (w/o vs. CHQ; Fig. 4A and B). In contrast, incubation

with proteasome inhibitors did not prevent the reduction in KCTD16 expression induced by 14-3-3 γ (~30-40% down-regulation of KCTD16 in the presence of DMSO, EPO and MG). These data suggest that 14-3-3 γ promotes KCTD16 degradation through the lysosome. It is interesting to note that in the absence of 14-3-3 γ the expression of KCTD16 increased ~2 fold when applying EPO or MG (Fig. 4C), which indicates proteasomal degradation of KCTD16. The expression of KCTD16 was unchanged upon CHQ incubation.

14-3-3 ε increases the stability of KCTD16 and its affinity to form tetramers

The ability of proteins to form dimers or oligomers is a well described mechanism to control protein stability and activity (Mateu, 2002, Li et al., 2006, Freeman et al., 2013). As KCTD proteins are a family of tetramerizing proteins, we investigated whether increased expression levels of KCTD16 induced by 14-3-3 ε correlate with higher stability of KCTD16 resulting from enhanced tetramerization. We used BRET donor saturation assays to study the formation of KCTD16 homo-tetramers. Binding of the donor fusion protein Rluc-KCTD16 to the acceptor fusion protein Venus-KCTD16 induces specific BRET that allows studying the affinity and degree of tetramerization. Expression of increasing amounts of Venus-KCTD16 with a fixed amount of Rluc-KCTD16 results in hyperbolic BRET donor saturation curves (Fig. 5A, up), consistent with KCTD16 being able to form tetramers as described earlier (Schwenk et al., 2010). When co-expressing fixed amounts of 14-3-3 ε we could still observe a specific BRET donor saturation curve suggesting that 14-3-3 ε does not prevent KCTD16 tetramer formation. However, compared to experiments without 14-3-3 ε, the BRET donor saturation curves were shifted towards (i) lower half-maximal BRET ($BRET_{50}$) and (ii) lower maximal BRET ($BRET_{max}$). The $BRET_{50}$ reflects how much BRET acceptor is required to reach 50% of the $BRET_{max}$ signal. Therefore, a left shift of the BRET donor saturation curve, as observed, is indicative for an increased affinity between the donor fusion protein and the acceptor fusion protein. At the same time, the decreased $BRET_{max}$ likely reflects a conformational rearrangement in the KCTD16 tetramer upon 14-3-3 ε binding. To test whether these effects arise specifically from direct interaction between KCTD16 and 14-3-3 ε, we conducted the same BRET donor saturation curves with Rluc-KCTD12 and Venus-KCTD12. As expected, we obtained hyperbolic BRET donor saturation curves confirming that also KCTD12 forms tetramers (Fig. 5A, down). In the presence of 14-3-3 ε, the BRET donor saturation curves were neither shifted in $BRET_{max}$ nor in $BRET_{50}$ suggesting that 14-3-3 ε specifically increases the affinity to form tetramers for its binding partner KCTD16 but not for non-interacting KCTD proteins.

To address the question whether 14-3-3 ε increases the amount of KCTD16 tetramers, we co-immunoprecipitated myc-KCTD16 with flag-KCTD16 in the presence and absence of 14-3-3 ε. Myc- and flag-tagged KCTD16 were enriched in the input as well as in the immunoprecipitation (Fig. 5B), which suggests that 14-3-3 ε increases the amount of tetramerized KCTD16. Additionally, we performed Native PAGE experiments to study if (i) KCTD16 per se exists as monomer and (ii) if 14-3-3 ε co-expression raises the amount of KCTD16 tetramers relative to KCTD16 monomers. Under native conditions, co-expression of 14-3-3 ε increased the amount of KCTD16 tetramers (Fig. 5C). However, KCTD16 seems to exist exclusively as tetramer as no monomer signal was observed. Altogether, these data indicate that 14-3-3 ε increases the stability of KCTD16 by enhancing its affinity to form tetramers.

KCTD16 links 14-3-3 proteins to $GABA_B$ receptors, which consequently regulates the expression of the $GABA_{B2}$ subunit

Distinct 14-3-3 isoforms, ζ and η, are known interactors of the $GABA_{B1}$ subunit (Couve et al., 2001, Brock et al., 2005, Laffray et al., 2012). Still, no 14-3-3 isoforms were yet shown to interact with the $GABA_{B2}$ subunit. Based on our findings that 14-3-3 ε and γ are binding partners of KCTD16, we asked the question whether the same 14-3-3 isoforms associate with the $GABA_{B2}$ subunit as well or whether they are part of the receptor complex solely in the presence of KCTD16. We found that both 14-3-3 ε and γ did not directly interact with the $GABA_{B2}$ subunit (Fig. 6A, up). However, when KCTD16 was co-expressed, 14-3-3 ε and γ were able to co-precipitate both KCTD16 and the $GABA_{B2}$ subunit (Fig. 6A, bottom) indicating that they are part of the receptor complex in the presence of KCTD16. Furthermore, when co-expressing KCTD16, both 14-3-3 isoforms regulated the expression of the $GABA_{B2}$ subunit in the same way as they regulate the expression of KCTD16 (Fig. 6B). These data suggest that 14-3-3 ε and γ are part of the $GABA_B$ receptor/KCTD16 complex and that they simultaneously regulate the expression of KCTD16 and $GABA_{B2}$.

14-3-3 proteins regulate the Gα-signaling of $GABA_B$/KCTD16 receptors

Cell surface targeting of $GABA_B$ receptors is a precisely regulated process (Margeta-Mitrovic et al., 2000, Calver et al., 2001, Pagano et al., 2001). Whereas $GABA_{B1}$ homodimers are retained in the ER through their ER retention signal, $GABA_{B1/2}$ heterodimers reach the cell surface and form functional receptors. The expression of the $GABA_{B2}$ subunit determines therefore the rate of functional receptors at the cell membrane. To test if 14-3-3 proteins control the level of functional membrane $GABA_B$ receptors we investigated whether the signaling of $GABA_B$ receptors is changed in the presence of 14-3-3 proteins. The Gα-signaling of $GABA_B$ receptors can be monitored by a protein-fragment complementation (PLC) assay in which split variants of the Renilla luciferase are fused to the regulatory (R) and the catalytic (C) subunit of the protein kinase-A (PKA) (Fig. 7A). High bioluminescence corresponds to inactive PKA composed of an R/C subunit heterodimer. High intracellular cAMP levels induce the dissociation of the R subunit from the C subunit leading to the activation of PKA, which accordingly corresponds to low bioluminescence. As $GABA_B$ receptors are $G\alpha_{i/o}$-coupled receptors, applying baclofen to CHO-K1 cells stably expressing $GABA_{B1}$ and $GABA_{B2}$ subunits results in PKA inhibition and hence to an increase in bioluminescence (Fig. 7B). Co-expression of 14-3-3 ε, KCTD16 or KCTD16 together with 14-3-3 ε significantly increased the Gα-signaling of $GABA_B$ receptors (Fig. 7C). In contrast, co-expression of KCTD16 together with 14-3-3 γ decreased the Gα-signaling of $GABA_B$ receptors whereas co-expression of 14-3-3 γ alone was without effect. These data suggest that 14-3-3 proteins regulate the expression of functional $GABA_B$ receptors at the cell surface to control $GABA_B$ receptor signaling in the presence of KCTD16.

14-3-3 proteins affect the binding of KCTD16 to CUL3

Some members of the KCTD protein family were reported to bear cullin adaptor function (Bayon et al., 2008, Azizieh et al., 2011, Correale et al., 2011, De Smaele et al., 2011, Balasco et al., 2014). It was not shown yet that the $GABA_B$ receptor interacting KCTD proteins 8, 12 and 16 share this function. To answer this question we expressed KCTD 8, 12 and 16 (KCTD16 alone and in the presence of 14-3-3 ε or γ) in COS-1 cells and performed immunoprecipitations against endogenous CUL3. We found that CUL3 was able to co-precipitate KCTD8 as well as KCTD16 (Fig. 8A). In the presence of 14-3-3 ε and γ,

the intensity of co-precipitated KCTD16 with CUL3 was greatly reduced (Fig. 8B). When normalizing the amount of co-precipitated KCTD16 to the input of KCTD16 it becomes obvious that 14-3-3 ε almost fully blocks KCTD16-CUL3 binding (Fig. 8C). Together, these data suggest that KCTD16 serves as substrate adaptor for CUL3 and that this function is regulated by 14-3-3 proteins.

DISCUSSION

Members of the KCTD protein family (8, 12, 12b, and 16) were reported to be auxiliary subunits of the $GABA_B$ receptor with KCTD12 being the most studied KCTD protein. It has been suggested that KCTD12 renders $GABA_B$-mediated signaling more precisely as it promotes fast desensitization of $GABA_B$-mediated responses but at the same time increases cell surface expression and stability of $GABA_B$ receptors (Schwenk et al., 2010, Ivankova et al., 2013, Adelfinger et al., 2014). KCTD8 was recently shown to reduce the basal G-protein activation mediated by $GABA_B$ receptors but not by other GPCRs like adenosine receptors 1 (Rajalu et al., 2014). Here, we identified members of the 14-3-3 protein family as a class of interaction partners that specifically bind to KCTD16, which might help to elucidate the function of KCTD16 on $GABA_B$ receptor signaling. The 14-3-3 isoforms ε and γ bind to the H2-domain of KCTD16 with each 14-3-3 isoform occupying a distinct binding site. Mutations of single serines or threonines in the H2-domain of KCTD16 did not abolish 14-3-3 interaction suggesting that 14-3-3 proteins have more than one binding site on KCTD16. Additionally, more studies are needed to elucidate whether 14-3-3 binding to KCTD16 is phosphorylation-dependent or -independent. Both 14-3-3 isoforms are associated specifically with the H2-domain of KCTD16 as they did not interact with KCTD8, which contains a H2-domain as well. The finding that the H2-domain harbors binding sites for other proteins - and 14-3-3s may not be the only binding partners - provides a first functional implication of the H2-domain besides its antagonizing effect on fast desensitization of $GABA_B$-activated K^+ currents (Seddik et al., 2012).

Moreover, we found that 14-3-3 binding affects the expression level of KCTD16. 14-3-3 proteins are well studied regulators of many eukaryotic proteins (for a review see (van Heusden, 2005)). They were shown to modulate protein localization, degradation, stability and activity as well as protein-protein interactions. This study shows that the co-expression of 14-3-3 γ shifts KCTD16 degradation from the proteasome to the lysosome. This switch might be mediated by 14-3-3 γ masking the PEST sequence that is localized in the H2-domain of KCTD16 (Suppl. fig. 1). The PEST sequence is a signal peptide responsible for protein degradation (Rogers et al., 1986) and would explain why KCTD16 shows a very low basal expression compared to KCTD12 or KCTD8 that do not contain such a sequence. Furthermore, it may be possible that 14-3-3 binding promotes the translocation of KCTD16 from the cytosol to the plasma membrane. Consequently, cytosolic KCTD16 would be rapidly degraded by the proteasome while membrane located KCTD16 - probably part of the $GABA_B$ receptor complex - would be degraded by the lysosome. This model would agree with earlier findings that KCTD proteins remain associated with the $GABA_B$ receptor during internalization (Ivankova et al., 2013).

On the other hand, 14-3-3 ε seemed to have a positive effect on KCTD16 expression. Co-expression of 14-3-3 ε strongly increased protein levels of KCTD16. This observation was not exceptional as 14-3-3 proteins were not only shown to affect the degradation of their binding partners but also to increase the stability or activity of their binding partners (Zheng et al., 2003, Winge et al., 2008, Woodcock et al., 2009, Barbash et al., 2011). $SCF^{Fbx}4$ activity, for example, is higher when interacting with 14-3-3 proteins as a result of augmented $SCF^{Fbx}4$ dimerization. Other 14-3-3 binding partners,

like AANAT and tryptophan hydroxylase, were found to form oligomers or contain dimer interfaces (Mockus et al., 1998, Khalil et al., 1999, Tenner et al., 2007) which might indicate that also these proteins are more stable upon 14-3-3-mediated dimerization although this is not proven yet. The results from BRET donor saturation assays in this study suggest that the effect of 14-3-3 ε on KCTD16 expression is mediated by enhancing the affinity of KCTD16 to form tetramers. In this assay, 14-3-3 ε did not elevate KCTD16 expression levels, yet shifting the binding curves for KCTD16 tetramers towards lower $BRET_{50}$ values. The lack of 14-3-3-mediated changes in KCTD16 levels might result as this assay was performed 24 h after transfection compared to the expression or co-immunoprecipitation studies that were done 48 h after transfection. Co-immunoprecipitations and Native PAGE experiments support the hypothesis of 14-3-3 ε enhancing KCTD16 self-assembly (stronger signals of co-precipitated KCTD16 in Figure 5B and of tetrameric KCTD16 in Figure 5C). The detection of non-denatured KCTD16 in the presence of 14-3-3 ε reveals a defined signal at the height of ~220 kDa correlating to the height of a KCTD16 tetramer while the signal is more diffuse towards bigger complexes in the absence of 14-3-3 ε. This suggests that KCTD16 is able to build higher forms of multimers but the formation of distinct tetramers is promoted by 14-3-3 ε. Native PAGE experiments further indicate that KCTD16 forms exclusively multimers due to the lack of monomeric KCTD16 in Figure 5C.

Until now, KCTD16 could not be assigned to a distinct function in $GABA_B$ signaling. This study reveals that KCTD16 serves as linker between the $GABA_B$ receptor and 14-3-3 proteins, which enables the regulation of $GABA_{B2}$ expression mediated by 14-3-3 proteins. This regulation very likely affects the number of cell surface receptors as the Gα-mediated signaling of $GABA_B$ receptors was found to be altered upon KCTD16 and 14-3-3 co-expression. The results of the PKA PCA assay are in accordance with the results of the Western blot analysis. In the presence of KCTD16, 14-3-3 ε up-regulates $GABA_{B2}$ expression as well as $GABA_B$ receptor-mediated PKA inhibition while 14-3-3 γ has the opposite effect. However, co-expression of 14-3-3 ε alone already increased the Gα-signaling of $GABA_B$ receptors. In contrast to 14-3-3 γ, which forms both homodimers and heterodimers preferentially with 14-3-3 ε, 14-3-3 ε does not form homodimers but heterodimers with most other 14-3-3 isoforms (Chaudhri et al., 2003). As CHO cells endogenously express 14-3-3 proteins (Feng et al., 2000, Rowland et al., 2011), heterodimerization of 14-3-3 ε with other 14-3-3 isoforms might give unspecific effects. Furthermore, $GABA_B$ receptors containing KCTD16 showed more inhibition of PKA compared to receptors without KCTD16 (Fig. 7B-C). This result might indicate that either endogenously expressed 14-3-3 ε is sufficient to display the same effect as overexpression of 14-3-3 ε or that KCTD16 alone increases the amount of cell surface $GABA_B$ receptors. Previous data show already a slight increase in cell surface $GABA_B$ receptors when co-expressed with KCTD16 (Ivankova et al., 2013). The Gα-signaling of $GABA_B$ receptors containing KCTD16 is not potentiated in the presence of 14-3-3 ε, which would indicate that PKA is already fully inhibited by $GABA_B$ receptors in the presence of either KCTD16 or 14-3-3 ε.

KCTD proteins belong to the family of BTB (bric-a-brac, tramtrak and broad complex)/POZ (poxvirus zinc finger) domain-containing proteins. Some of those members have been shown to serve as substrate adaptors for cullin ubiquitin ligases. KCTD5, 6, 7, 11 and 21 were identified to interact with CUL3, one of seven cullins that is known to bind BTB proteins (Furukawa et al., 2003, Geyer et al., 2003, Pintard et al., 2004, Bayon et al., 2008, Canettieri et al., 2010, Azizieh et al., 2011, Correale et al., 2011, De Smaele et al., 2011, Balasco et al., 2014). Here we show that KCTD16, and further KCTD8, is able to bind CUL3 which suggests a role for KCTD16 as a substrate adaptor for CUL3. However the substrate is not known yet, it is tempting to postulate that $GABA_B$ receptors might be

the substrate for the CUL3-KCTD16 complex as some steps of $GABA_B$ receptor degradation are still unknown. $GABA_B$ receptor degradation is initiated through two major pathways. Newly synthetized receptors in the ER are degraded by the endoplasmic reticulum-associated degradation (ERAD) (Zemoura et al., 2013). Misfolded $GABA_B$ receptors are K48-linked polyubiquitinated on $GABA_{B2}$-K767/771 by the ERAD E3 ubiquitin ligase Hrd1 and constitutively directed to proteasomal degradation. The ERAD-mediated degradation of $GABA_B$ receptors determines the cell surface expression of functional receptors as it limits the forward trafficking of receptors to the plasma membrane. Cell surface $GABA_B$ receptors on the other hand are constitutively internalized via the dynamin- and clathrin-dependent pathway to be recycled in endosomes or eventually degraded in lysosomes (Grampp et al., 2007, Grampp et al., 2008). The phosphorylation of distinct sites on $GABA_{B1}$ and $GABA_{B2}$ determines whether $GABA_B$ receptors are directed to endosomes or lysosomes (Guetg et al., 2010, Terunuma et al., 2010). However, it is not known yet how $GABA_B$ receptors are designated for lysosomal degradation. In general, K63-linked polyubiquitination was shown to stimulate endocytosis and subsequent degradation (Galan and Haguenauer-Tsapis, 1997). The analysis of the brain ubiquitome revealed two putative ubiquitination sites on the $GABA_{B2}$ subunit (Na et al., 2012). One of them, K771, was already confirmed to be K48-linked polyubiquitinated as described above (Zemoura et al., 2013). The other site, K800, has not been studied yet and it is possible that this site is K63-linked polyubiquitinated to serve as a signal for lysosomal degradation. In principal, K63-linked polyubiquitination can be achieved by CUL3 (Jin et al., 2009, Han et al., 2010), which would fit to our hypothesis that $GABA_B$ receptors are substrates of CUL3.

In conclusion, we propose that the complex formation of KCTD16 with 14-3-3 proteins determines the localization and fate of KCTD16 and the amount of functional $GABA_B$ receptors in the cell surface (Fig. 9). In the absence of 14-3-3 proteins and $GABA_B$ receptors, KCTD16 is mostly localized in the cytosol and rapidly degraded by the proteasome via its PEST-sequence. In the presence of $GABA_B$ receptors, KCTD16 is part of the receptor complex and located at the cell surface. 14-3-3 proteins may strengthen this interaction by either promoting the translocation of KCTD16 from the cytosol to the cell membrane or by stabilizing the binding through conformational changes. Dependent on the 14-3-3 isoform, $GABA_B$ receptors and KCTD16 are either stabilized or degraded. 14-3-3 γ enhances lysosomal degradation of the $GABA_B$-KCTD16 complex by cullin-mediated ubiquitination of the $GABA_B$ receptor. In contrast, 14-3-3 ε binding to KCTD16 stabilizes the $GABA_B$-KCTD16 complex. It promotes KCTD16 stability by increasing the affinity for KCTD16 tetramer formation while at the same time interfering with the binding of KCTD16 to CUL3. This in turn increases cell surface $GABA_B$ receptor levels resulting in elevated receptor signaling. Together, this model would show for the first time how cell surface $GABA_B$ receptors are regulated by their auxiliary subunit KCTD16 and how they are targeted to lysosomes for degradation. It further includes that the lysosomal degradation of $GABA_B$ receptors is highly dependent on the expression pattern of KCTD proteins as well as 14-3-3 proteins.

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Zheng W, Zhang Z, Ganguly S, Weller JL, Klein DC, Cole PA (2003) Cellular stabilization of the melatonin rhythm enzyme induced by nonhydrolyzable phosphonate incorporation. Nature structural biology 10:1054-1057.

FIGURE LEGENDS

FIGURE 1. **KCTD16 associates with 14-3-3 proteins through its C-terminal H2-domain.** Direct cell lysates (input) and co-immunoprecipitations of COS-1 cells transfected with 14-3-3 proteins and wild-type or chimeric KCTD proteins. KCTD proteins were immunoprecipitated with myc-, flag- or KCTD specific-antibodies as indicated. (A) Schematic figure showing the full-length KCTD proteins 12, 8, and 16 with their T1-, H1-, and H2-domain. KCTD12 lacks the H2-domain. The BTB/POZ domain is indicated in blue. (B) 14-3-3 γ and ε co-precipitate with KCTD16 but not with KCTD12. The addition of the H2-domain of KCTD16 to KCTD12 (myc-12-16H2) results in 14-3-3 binding, whereas the deletion of the H2-domain in KCTD16 (myc-16ΔH2) leads to 14-3-3 un-binding. (C) The isolated H2-domain of KCTD16 (myc-16H2) is sufficient to bind 14-3-3 proteins. The *asterisk* likely indicates an unspecific signal in the input that is absent in the immunoprecipitation. (D) 14-3-3 proteins bind specifically to the H2-domain of KCTD16 as they do not interact with KCTD8. IP, immunoprecipitation.

FIGURE 2. **Mapping of 14-3-3 binding sites in the H2-domain of KCTD16.** Direct cell lysates (input) and co-immunoprecipitations of COS-1 cells transfected with 14-3-3 proteins and chimeric KCTD proteins. KCTD proteins were immunoprecipitated with myc- or KCTD12 specific-antibodies as indicated. (A) Schematic figure showing the chimeric KCTD proteins. The H2-domain of KCTD16 was added to the C-terminus of KCTD12 (12-16H2). Based on this construct, the following deletion mutants were generated: 12-16H2Δ60 (Δ416-475), 12-16H2Δ39 (Δ437-475) and 12-16H2ΔN (Δ328-362). (B) 14-3-3 γ and ε co-precipitate with the N-terminal deletion mutant (12-16H2ΔN) but not with the mutant lacking half of the H2-domain (12-16H2Δ60). (C) 14-3-3 γ, but not 14-3-3 ε, co-precipitates with the C-terminal deletion mutant 12-16H2Δ39. IP, immunoprecipitation.

FIGURE 3. **14-3-3 proteins regulate the expression level of KCTD16.** Direct cell lysates of COS-1 cells transfected with 14-3-3 proteins and KCTD16 (A), KCTD12 (B), 16H2Δ39 (C) or 16H2Δ60 (D). The binding of 14-3-3 ε increases the expression of its interaction partner KCTD16 (A), whereas the binding of 14-3-3 γ decreases the expression of its interaction partners KCTD16 (A) and 16H2Δ39 (C). Binding-deficient KCTDs, like KCTD12 (B), 16H2Δ39 (deficient in 14-3-3 ε binding, C) or 16H2Δ60 (deficient in 14-3-3 ε and γ binding, D) do not show changes in expression upon 14-3-3 protein co-expression. Blots are representative of 3-4 independent experiments. Bar graphs on the right summarize the amount of KCTD expression (in %) normalized to the loading control actin on the same blot. Data are means ± *SEM*, n = 3-4. *, $p < 0.05$; **, $p < 0.01$; *t* test.

FIGURE 4. **14-3-3 γ promotes lysosomal degradation of KCTD16.** Direct cell lysates of COS-1 cells expressing KCTD16 with and without 14-3-3 γ. Cells were untreated (lane 1 and 2, w/o, as a control for CHQ) or treated for 18 h with the lysosome-inhibitor chloroquine (100 µM, lane 3 and 4, CHQ), DMSO (lane 5 and 6, as a control for EPO and MG) or the proteasome-inhibitors epoxomicine (2 µM, lane 7 and 8, EPO) or MG132 (10 µM, lane 9 and 10, MG). Adding the lysosome-inhibitor blocks 14-3-3 γ-mediated knock-down of KCTD16 expression. Increased total ubiquitin levels ensure the effectiveness of the treatment. Blots are representative of four independent experiments. The bar graph summarizes the amount of KCTD16 expression (in %) normalized to the loading control actin on the same blot. Data are means ± *SEM*, n = 4. *, $p < 0.05$; **, $p < 0.01$; *t* test.

FIGURE 5. **14-3-3 ε increases the affinity for KCTD16 to form tetramers.** (A) BRET donor saturation curves from COS-1 cells expressing fixed amounts of Rluc-KCTD16 and increasing amounts of Venus-KCTD16 (up) or fixed amounts of Rluc-KCTD12 and increasing amounts of Venus-KCTD12 (down) in the presence (open circles) and absence (filled circles) of 14-3-3 ε. 14-3-3 ε affects the binding properties of KCTD16 - but not KCTD12 - to form tetramers. BRET is expressed as milli BRET units

(mBU) determined as net BRET × 1000. Data points are means ± *SEM* of technical quadruplicates of a representative experiment, *n* = 5-8. (B) Direct cell lysates (input) and co-immunoprecipitations of COS-1 cells expressing flag- and myc-KCTD16 with and without 14-3-3 ε. In the presence of 14-3-3 ε, the amount of co-immunoprecipitated myc-KCTD16 with flag-KCTD16 is increased. (C) Native PAGE of cell lysates from COS-1 cells expressing myc-KCTD16 with and without 14-3-3 ε. The amount of KCTD16 tetramers is increased when 14-3-3 ε is co-expressed. KCTD16 can be detected in a high molecular weight complex. 14-3-3 ε can be detected as monomer and in a high molecular weight complex. A short (left) and long (right) exposure of the immunoblot against myc is shown. IP, immunoprecipitation; IB, immunoblot.

FIGURE 6. **KCTD16 links 14-3-3 proteins to $GABA_B$ receptors, which leads to changes in $GABA_{B2}$ expression.** Direct cell lysates (input) and co-immunoprecipitations (A) and direct cell lysates (B) of COS-1 cells transfected with 14-3-3 proteins and $GABA_{B2}$ (GB2) with and without KCTD16. 14-3-3 proteins were immunoprecipitated with myc- or HA-antibodies as indicated. (A) 14-3-3 proteins do not directly interact with $GABA_{B2}$ (up) but are part of the $GABA_B$ receptor complex in the presence of KCTD16 (down). (B) 14-3-3 proteins slightly decrease the expression of $GABA_{B2}$ in the absence of KCTD16. In the presence of KCTD16, 14-3-3 proteins show the same regulation of $GABA_{B2}$ expression as observed for KCTD16 expression. Blots are representative of 6-14 independent experiments. The bar graphs on the right summarize the amount of $GABA_{B2}$ expression normalized to the loading control actin on the same blot. Data are means ± *SEM*, *n* = 6-14. *, $p < 0.05$; *t* test.

FIGURE 7. **14-3-3 proteins affect the Gα-signaling of $GABA_B$ receptors.** (A) Scheme of the protein-fragment complementation assay. Split variants of the Rluc-donor are fused to the regulatory and the catalytic subunit (R and C) of the protein kinase A (PKA). High bioluminescence results from low PKA activity due to the tetrameric structure of the kinase. Increasing intracellular cAMP levels caused by forskolin (FSK) activates PKA leading to a dissociation of R and C, which results in low bioluminescence. (B) Real-time kinetics (normalized to control Reg/Reg) in response to baclofen (100 µM) of Reg/Cat recorded from CHO-K1 cells stably expressing $GABA_{B1}$ and $GABA_{B2}$ and transiently expressing pCI-empty (w/o), 14-3-3 ε, 14-3-3 γ, KCTD16 or the combinations KCTD16 + 14-3-3 ε, and KCTD16 + 14-3-3 γ. Increased PKA inhibition is detected with 14-3-3 ε, KCTD16 and KCTD16 + 14-3-3 ε. Decreased PKA inhibition is detected with KCTD16 + 14-3-3 γ. Changes in bioluminescence are expressed in relative luminescence (in % of Reg/Reg). One representative experiment is shown. (C) The bar graph summarizes the changes of Reg/Cat (in % of Reg/Reg). Data are means ± *SEM*, *n* = 4. *, $p < 0.05$; **, $p < 0.01$; *t* test.

FIGURE 8. **KCTD16-CUL3 interaction is reduced in the presence of 14-3-3 proteins.** (A) Direct cell lysates (input) and co-immunoprecipitations of COS-1 cells expressing KCTD proteins and 14-3-3 proteins as indicated. Endogenous CUL3 was immunoprecipitated with a cullin3-specific antibody. KCTD16 and KCTD8 co-precipitated with CUL3. The amount of co-precipitated KCTD16 is decreased in the presence of 14-3-3 proteins. IP, immunoprecipitation. (B) The bar graph summarizes the amount of co-precipitated KCTD16 (in %). Data are means ± *SEM*, *n* = 2.

FIGURE 9. **Degradation pathway of cell surface $GABA_B$ receptors and KCTD16.** KCTD16 is a cytosolic protein that is rapidly degraded by the proteasome (1). Degradation is mediated by the PEST sequence in the H2-domain of KCTD16. The binding of 14-3-3 proteins masks the PEST sequence in KCTD16, which inhibits proteasomal degradation of KCTD16 (2). KCTD16 harbors the function as an adaptor protein for cullin3 (CUL3). The binding of the KCTD16-CUL3 complex to $GABA_B$ receptors (3) leads to ubiquitination and subsequent degradation of the receptor (4). This process is limited by low

expression of KCTD16 due to its rapid proteasomal degradation. Binding of 14-3-3 γ to KCTD16 promotes the lysosomal degradation of $GABA_B$ receptors (5). As KCTD16 constitutively binds to $GABA_B$ receptors, its degradation is shifted from the proteasome to the lysosome. The binding of 14-3-3 ε to KCTD16 enhances the stability of the $GABA_B$/KCTD16 complex by interfering with the KCTD16-CUL3 complex (6).

SUPPL. FIGURE 1. **KCTD16 contains a PEST sequence.** (A) Schematic figure showing the PEST sequence in KCTD16 and its conservation throughout different species. The PEST sequence was identified using emboss.bioinformatics.nl.

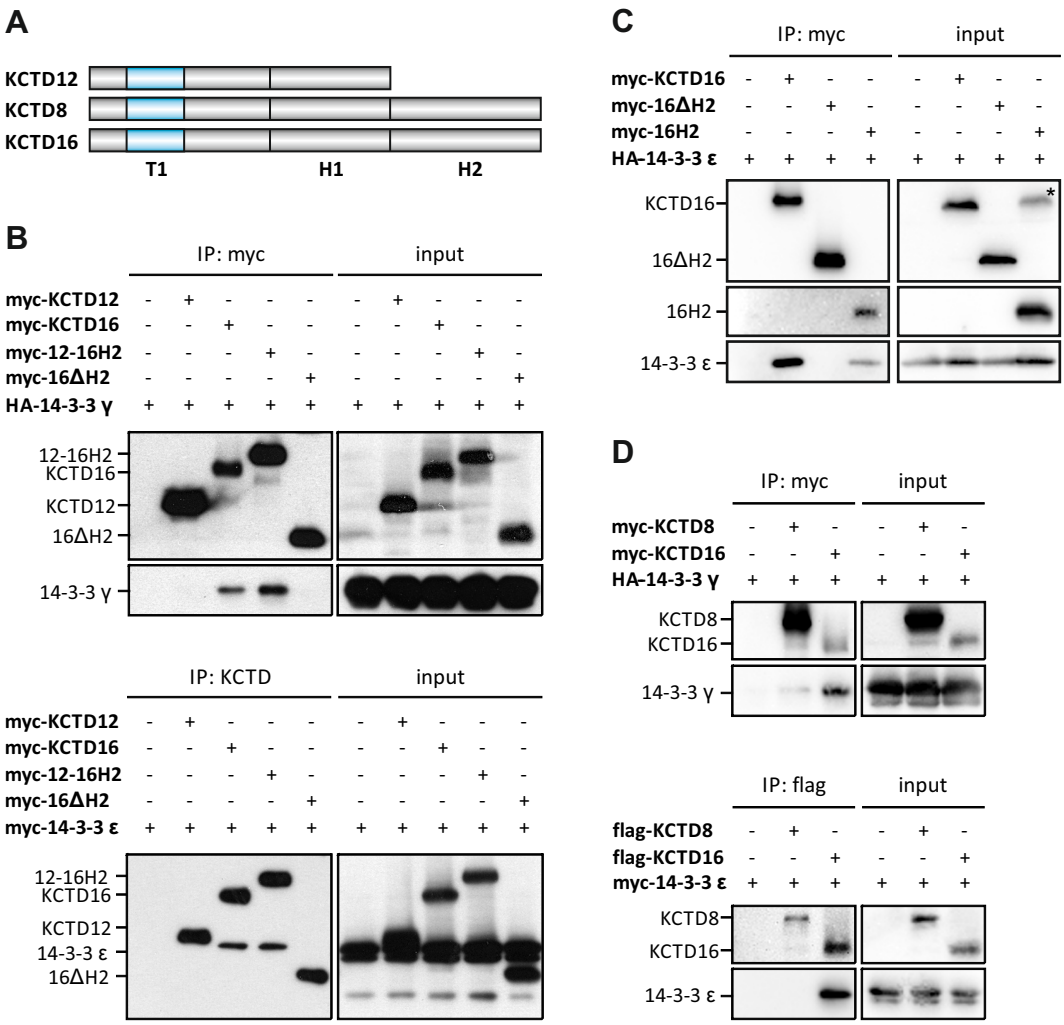

A
KCTD12
KCTD8
KCTD16
T1
H1
H2
B
IP: myc
input
myc-KCTD12
myc-KCTD16
myc-12-16H2
myc-16ΔH2
HA-14-3-3 γ
12-16H2
KCTD16
KCTD12
16ΔH2
14-3-3 γ
IP: KCTD
input
myc-KCTD12
myc-KCTD16
myc-12-16H2
myc-16ΔH2
myc-14-3-3 ε
12-16H2
KCTD16
KCTD12
14-3-3 ε
16ΔH2
C
IP: myc
input
myc-KCTD16
myc-16ΔH2
myc-16H2
HA-14-3-3 ε
KCTD16
16ΔH2
16H2
14-3-3 ε
*
D
IP: myc
input
myc-KCTD8
myc-KCTD16
HA-14-3-3 γ
KCTD8
KCTD16
14-3-3 γ
IP: flag
input
flag-KCTD8
flag-KCTD16
myc-14-3-3 ε
KCTD8
KCTD16
14-3-3 ε

FIGURE 1

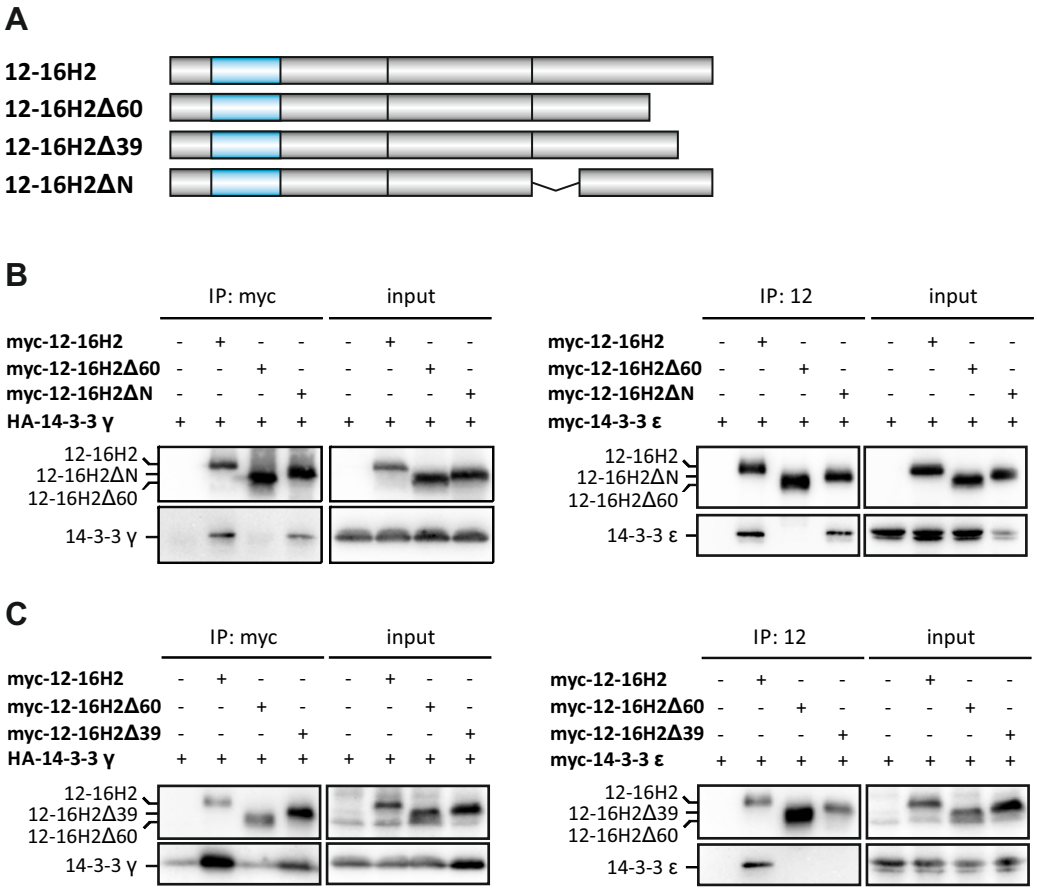

A
12-16H2
12-16H2Δ60
12-16H2Δ39
12-16H2ΔN
B
IP: myc
input
myc-12-16H2
myc-12-16H2Δ60
myc-12-16H2ΔN
HA-14-3-3 γ
12-16H2
12-16H2ΔN
12-16H2Δ60
14-3-3 γ
IP: 12
input
myc-12-16H2
myc-12-16H2Δ60
myc-12-16H2ΔN
myc-14-3-3 ε
12-16H2
12-16H2ΔN
12-16H2Δ60
14-3-3 ε
C
IP: myc
input
myc-12-16H2
myc-12-16H2Δ60
myc-12-16H2Δ39
HA-14-3-3 γ
12-16H2
12-16H2Δ39
12-16H2Δ60
14-3-3 γ
IP: 12
input
myc-12-16H2
myc-12-16H2Δ60
myc-12-16H2Δ39
myc-14-3-3 ε
12-16H2
12-16H2Δ39
12-16H2Δ60
14-3-3 ε

FIGURE 2

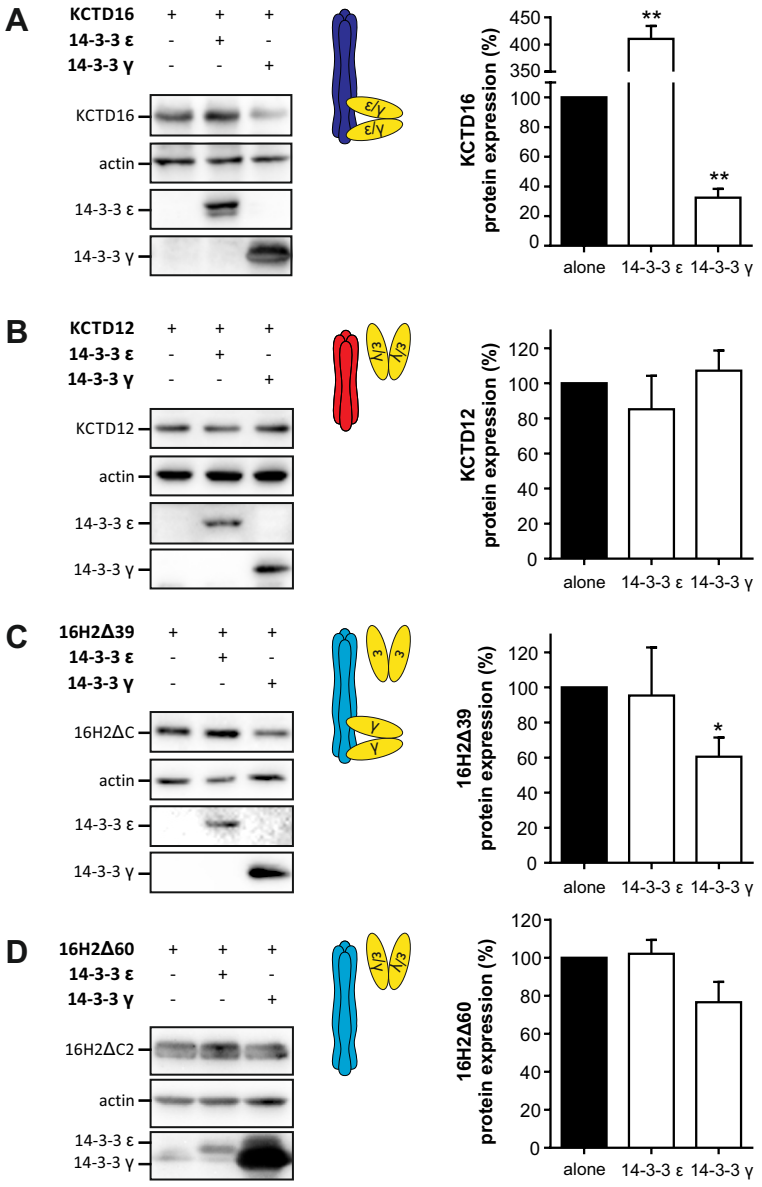

A
KCTD16 + + +
14-3-3 ε - + -
14-3-3 γ - - +
KCTD16
actin
14-3-3 ε
14-3-3 γ
ε/γ
ε/γ
KCTD16 protein expression (%)
450
400
350
100
80
60
40
20
0
**
**
alone
14-3-3 ε
14-3-3 γ
B
KCTD12 + + +
14-3-3 ε - + -
14-3-3 γ - - +
KCTD12
actin
14-3-3 ε
14-3-3 γ
ε/ζ
ε/ζ
KCTD12 protein expression (%)
120
100
80
60
40
20
0
alone
14-3-3 ε
14-3-3 γ
C
16H2Δ39 + + +
14-3-3 ε - + -
14-3-3 γ - - +
16H2ΔC
actin
14-3-3 ε
14-3-3 γ
ζ
ζ
γ
γ
16H2Δ39 protein expression (%)
120
100
80
60
40
20
0
*
alone
14-3-3 ε
14-3-3 γ
D
16H2Δ60 + + +
14-3-3 ε - + -
14-3-3 γ - - +
16H2ΔC2
actin
14-3-3 ε
14-3-3 γ
ε/ζ
ε/ζ
16H2Δ60 protein expression (%)
120
100
80
60
40
20
0
alone
14-3-3 ε
14-3-3 γ

FIGURE 3

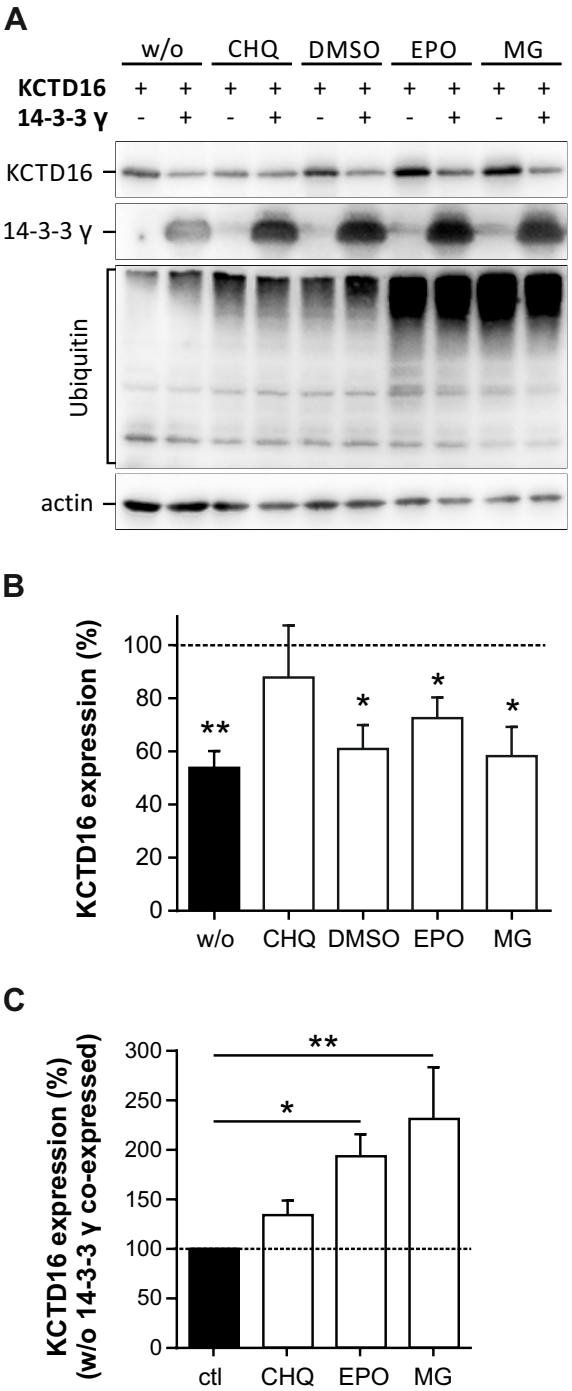

A
w/o
CHQ
DMSO
EPO
MG
KCTD16 + + + + + + + + + +
14-3-3 γ - + - + - + - + - +
KCTD16
14-3-3 γ
Ubiquitin
actin
B
KCTD16 expression (%)
100
80
60
40
20
0
**
*
*
*
w/o
CHQ
DMSO
EPO
MG
C
KCTD16 expression (%)
(w/o 14-3-3 γ co-expressed)
300
250
200
150
100
50
0
**
*
ctl
CHQ
EPO
MG

FIGURE 4

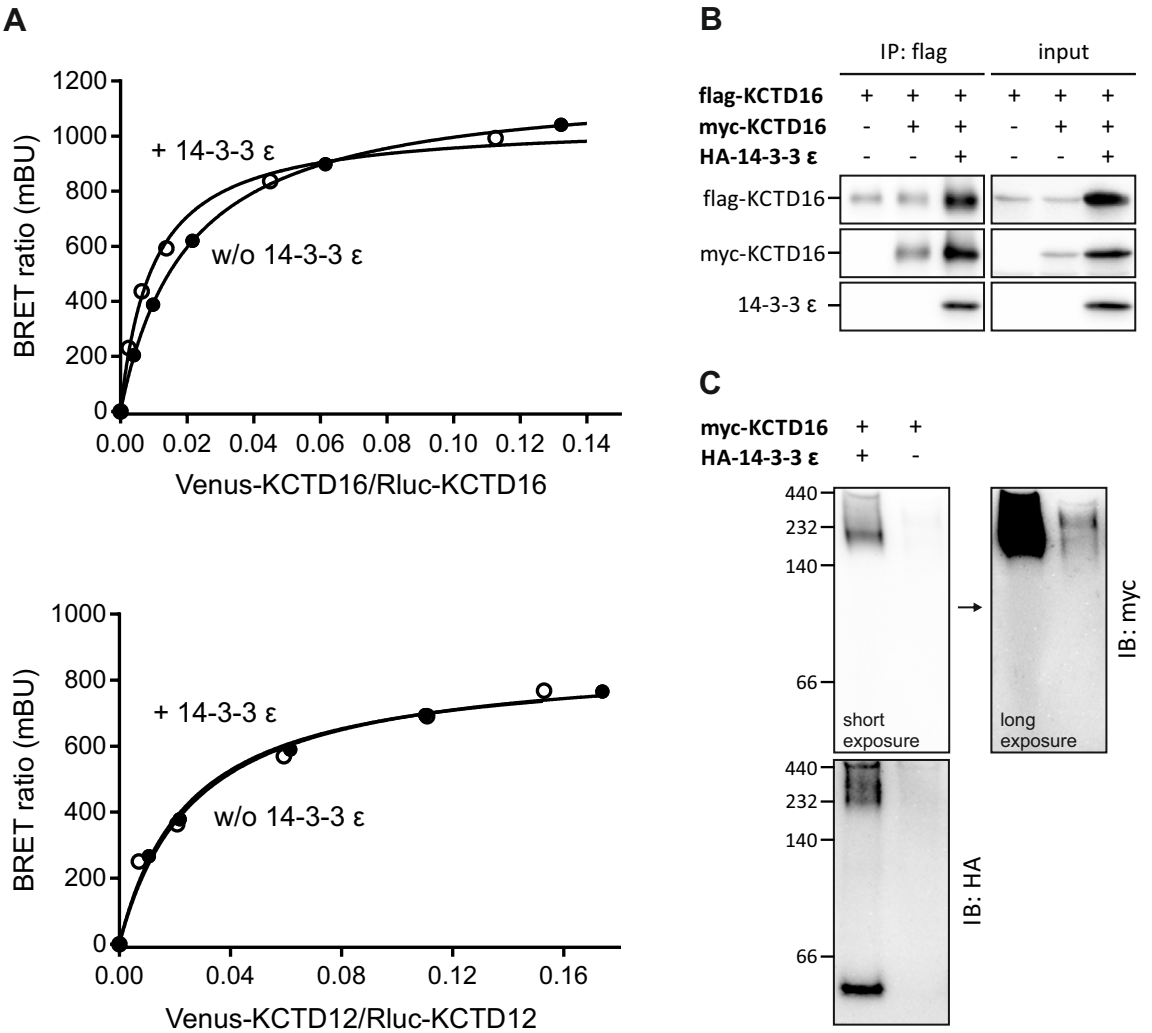

A
BRET ratio (mBU)
+ 14-3-3 ε
w/o 14-3-3 ε
Venus-KCTD16/Rluc-KCTD16
Venus-KCTD12/Rluc-KCTD12
B
IP: flag
input
flag-KCTD16
myc-KCTD16
HA-14-3-3 ε
14-3-3 ε
C
short exposure
long exposure
IB: myc
IB: HA

FIGURE 5

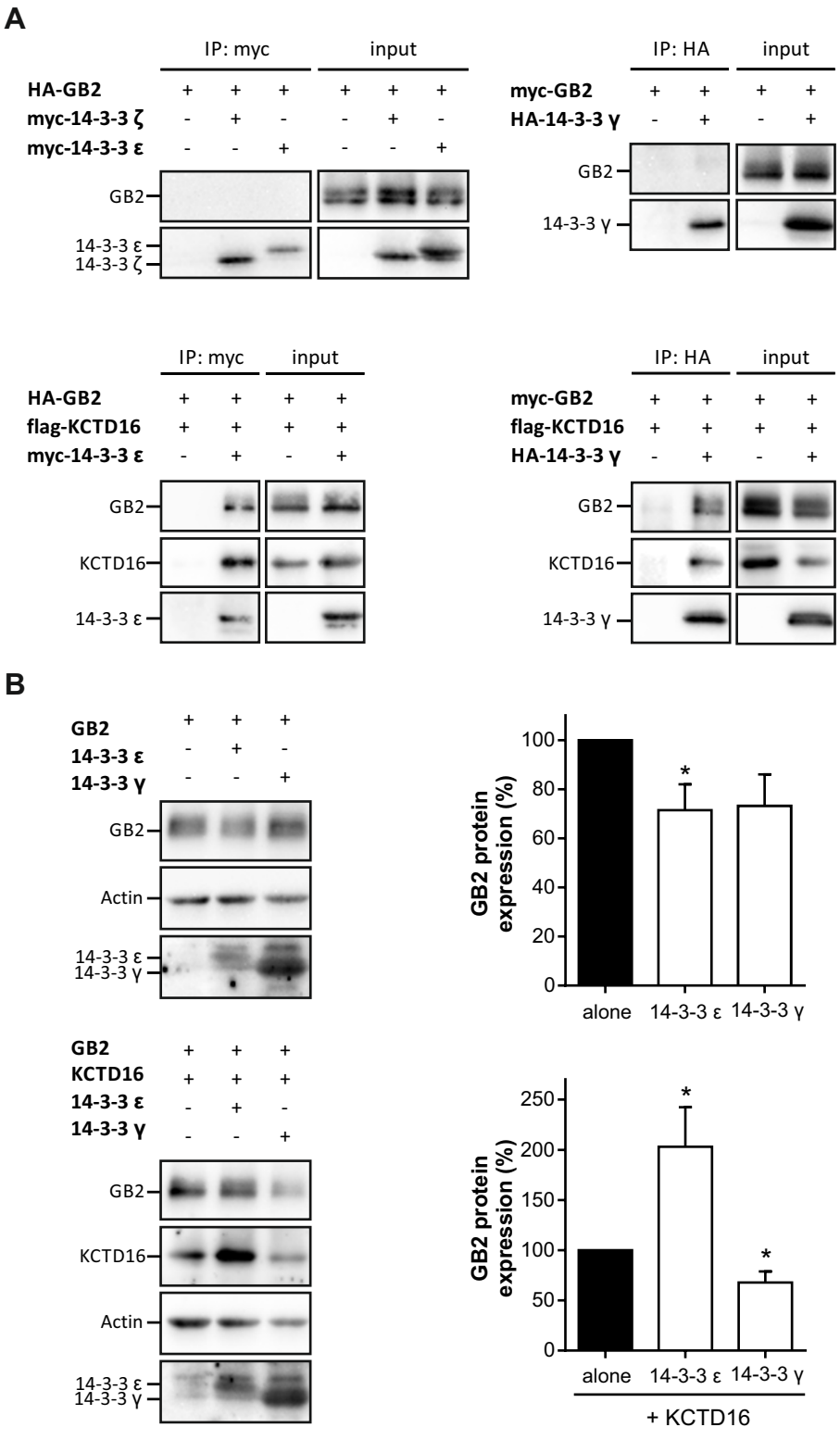

A
IP: myc
input
HA-GB2
myc-14-3-3 ζ
myc-14-3-3 ε
GB2
14-3-3 ε
14-3-3 ζ
IP: HA
input
myc-GB2
HA-14-3-3 γ
GB2
14-3-3 γ
IP: myc
input
HA-GB2
flag-KCTD16
myc-14-3-3 ε
GB2
KCTD16
14-3-3 ε
IP: HA
input
myc-GB2
flag-KCTD16
HA-14-3-3 γ
GB2
KCTD16
14-3-3 γ
B
GB2
14-3-3 ε
14-3-3 γ
GB2
Actin
14-3-3 ε
14-3-3 γ
GB2 protein expression (%)
alone
14-3-3 ε
14-3-3 γ
GB2
KCTD16
14-3-3 ε
14-3-3 γ
GB2
KCTD16
Actin
14-3-3 ε
14-3-3 γ
GB2 protein expression (%)
alone
14-3-3 ε
14-3-3 γ
+ KCTD16

FIGURE 6

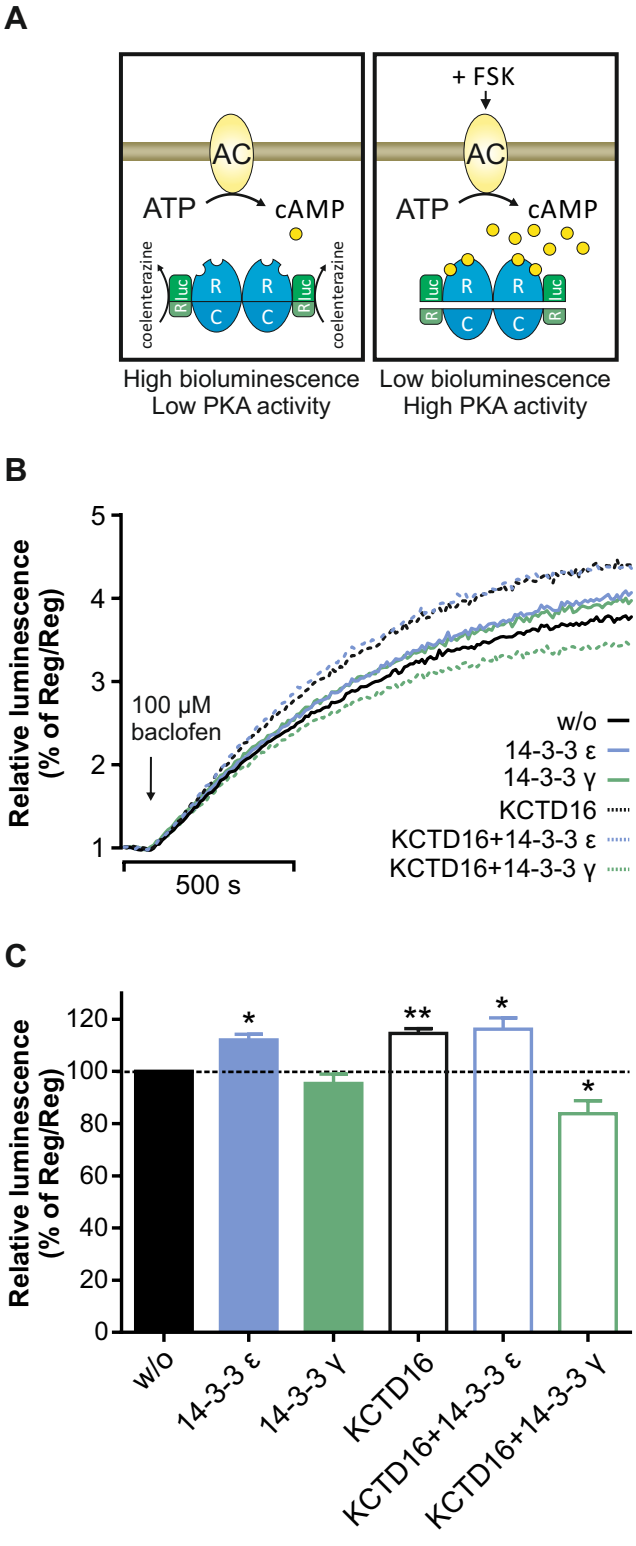

A
AC
ATP
cAMP
+ FSK
coelenterazine
Rluc
R
C
High bioluminescence
Low PKA activity
Low bioluminescence
High PKA activity
B
Relative luminescence
(% of Reg/Reg)
100 µM
baclofen
500 s
w/o
14-3-3 ε
14-3-3 γ
KCTD16
KCTD16+14-3-3 ε
KCTD16+14-3-3 γ
C
Relative luminescence
(% of Reg/Reg)
w/o
14-3-3 ε
14-3-3 γ
KCTD16
KCTD16+14-3-3 ε
KCTD16+14-3-3 γ

FIGURE 7

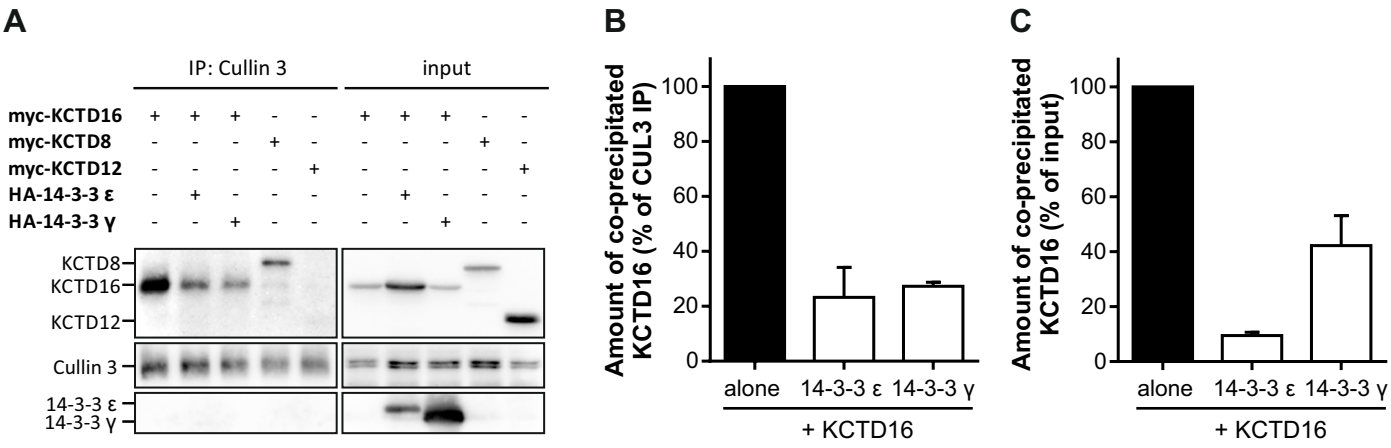

A
IP: Cullin 3
input
myc-KCTD16 + + + - - + + + - -
myc-KCTD8 - - - + - - - - + -
myc-KCTD12 - - - - + - - - - +
HA-14-3-3 ε - + - - - - + - - -
HA-14-3-3 γ - - + - - - - + - -
KCTD8
KCTD16
KCTD12
Cullin 3
14-3-3 ε
14-3-3 γ
B
Amount of co-precipitated KCTD16 (% of CUL3 IP)
100
80
60
40
20
0
alone
14-3-3 ε
14-3-3 γ
+ KCTD16
C
Amount of co-precipitated KCTD16 (% of input)
100
80
60
40
20
0
alone
14-3-3 ε
14-3-3 γ
+ KCTD16

FIGURE 8

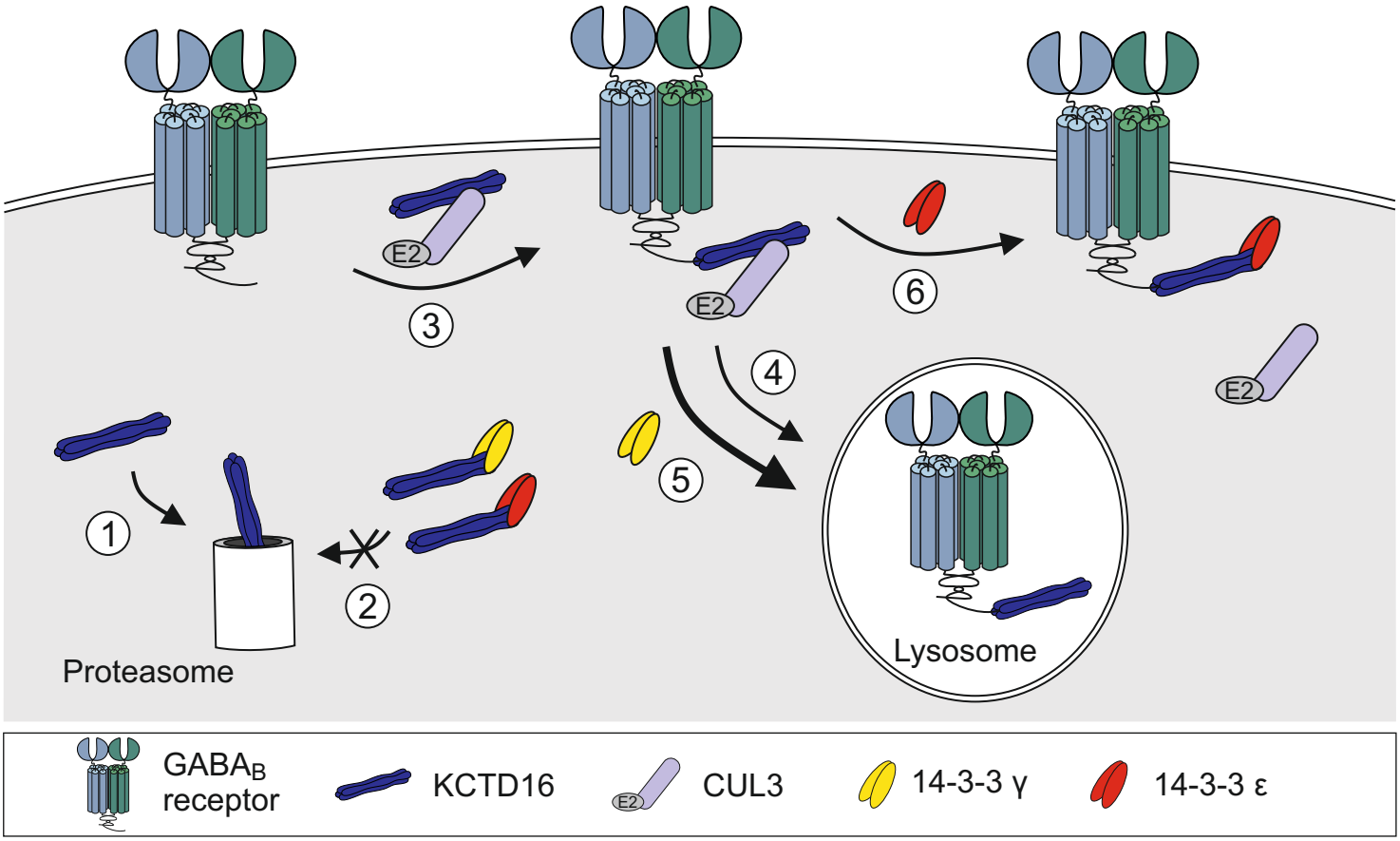
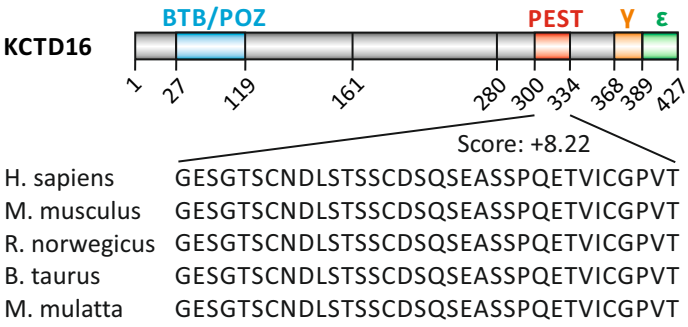

E2
3
E2
6
E2
4
5
1
2
Proteasome
Lysosome
GABA$_B$ receptor
KCTD16
CUL3
14-3-3 γ
14-3-3 ε

FIGURE 9



SUPPL. FIGURE 1

Table 1. Parameters from BRET saturation curves with Rluc-KCTD16/YFP-KCTD16 and Rluc-KCTD12/YFP-KCTD12.

BRET donor saturation assays were performed in COS-1 cells transfected with fixed amounts of HA-14-3-3 ε and Rluc-KCTD16/12 (donor) and increasing amounts of YFP-KCTD16/12 (acceptor). The $BRET_{max}$ is the maximal BRET signal obtained for a given donor/acceptor pair and the $BRET_{50}$ corresponds to the amount of acceptor needed to obtain 50% of the $BRET_{max}$. Data are presented as the mean ± *SEM* of the indicated number of independent experiments and were derived from data as presented in Fig. 5A. ***, $p < 0.001$ *, $p < 0.05$; compared to KCTD16, w/o 14-3-3 ε; *t* test.

	$BRET_{max}$	± *SEM*	$BRET_{50}$	± *SEM*	*n*
KCTD16, w/o 14-3-3 ε	1267.0	30.3	0.03317	0.00571	8
KCTD16, + 14-3-3 ε	1096.0***	18.4	0.01681*	0.00326	8
KCTD12, w/o 14-3-3 ε	900.5	24.4	0.02522	0.00535	5
KCTD12, + 14-3-3 ε	870.5	17.7	0.01972	0.00396	5

SUPPLEMENTARY TABLE 1: Antibody information

Antibody information		Application and corresponding figure	
Antibody and species	Product # and Supplier	IP	WB
anti-myc (mouse)	sc-40, Santa Cruz	1-1.5 µg (Protein G-Agarose or Dynabeads Protein-G) Figures: 1D-E, 2B-D, 3B	1:1000 Figures: 2B, 3B-C, 4A-D, 5, 6C, 7, 8 Suppl. fig. 1C
anti-myc (rabbit)	C3956, Sigma Aldrich	1 µg (Dynabeads Protein-G) Figures: 3C	1:1000 Figures: 1D-E, 2B-D, 3B, 6B
anti-HA (mouse)	MMS-101P, Covance	n/a	1:1000 Figures: 1D-E, 3C, 4A, 4C
anti-HA (rabbit)	RHGT-45A-Z, ICL	1 µg (Dynabeads Protein-G) Figures: 1D-E	1:1000 Figures: 2B, 2D, 3B
anti-HA (rat)	11867423001, Roche	n/a	1:1000 Figures: 2C, 4D, 5, 6B-C, 7, 8
anti-flag (mouse)	F1804, Sigma Aldrich	1-1.2 µg (Protein G-Agarose or Dynabeads Protein-G) Figures: 2D, 6B	1:1000 Figures: 1E
anti-flag (rabbit)	F7425, Sigma Aldrich	n/a	1:1000 Figures: 1E, 2D, 6B
anti-actin (mouse)	MAB1501, Millipore	n/a	1:1000 Figures: 4D, 5, 7
anti-actin (rabbit)	4970, Cell signaling	n/a	1:1000 Figures: 4A-C Suppl. fig. 1C
anti-KCTD16 (rabbit)	Metz et al., 2011	n/a	1:1000 Figures: 4A
anti-KCTD16 (guinea pig)	Metz et al., 2011	2.5 µl (Protein A-Agarose) Figures: 2B	n/a
anti-KCTD12 (rabbit)	Metz et al., 2011	2.5 µl (Dynabeads Protein-G) Figures: 3B-C	n/a
anti-KCTD12 (guinea pig)	Metz et al., 2011	2.5 µl (Protein A-Agarose) Figures: 2B	1:1000 Figures: 4B
anti-14-3-3 (rabbit)	sc-629, Santa Cruz	n/a	1:1000 Figures: 4B Suppl. fig. 1C
anti-ubiquitin (mouse)	BML-PW8810-0100, Enzo Life Science	n/a	1:1000 Figures: 5
anti-cullin 3 (rabbit)	ab75851, Abcam	2 µl (Dynabeads Protein-G) Figures: 8	1:1000 Figures: 8
anti-GABAB2 (rabbit)	AGB-002, Alomone	n/a	1:1000 Figures: 7
anti-GABAB2 (guinea pig)	AB5394, Millipore	n/a	1:2000 Figures: 1D-E,

IP, Immunoprecipitation; WB, Western blot

3 GENERAL DISCUSSION & FUTURE PERSPECTIVES

$GABA_B$ receptors play a fundamental role in modulating the synaptic transmission of inhibitory signals. Presynaptic $GABA_B$ receptors suppress evoked and spontaneous neurotransmitter release from synaptic clefts by inhibiting voltage-gated Ca^{2+} channels and adenylyl cyclase. Postsynaptic $GABA_B$ receptors decrease neuronal excitability by activating G protein-coupled inwardly rectifying potassium (GIRK) channels, which underlies the generation of slow inhibitory postsynaptic potentials. It is therefore of high importance to explore mechanisms that precisely control the activity of these receptors. Great interest lies in the fine-tuning of $GABA_B$ receptor-mediated signaling that allows slight changes in signal intensity dependent on localization, compartmentalization and complex composition. The discovery of auxiliary $GABA_B$ receptor subunits was already a significant progress in elucidating the discrepancies between recombinant and native $GABA_B$ receptor responses. And yet, a lot more mechanisms of receptor regulation are most likely unexplored until today. This dissertation aims to better understand the KCTD12-induced K^+ current desensitization and to describe new mechanisms that regulate the signaling of $GABA_B$ receptors.

The first part of this thesis unravels the mechanism of KCTD12-induced K^+ current desensitization. We show that this desensitization is activity-dependent, reversible, operates upstream of the GIRK channel and requires the interaction of KCTD12 with the G-protein. Although all KCTDs constitutively bind the G-protein and accelerate the rise time of $GABA_B$ responses, exclusively KCTD12 induces desensitization. We show that KCTD12, in contrast to KCTD8 and KCTD16, dynamically binds the G-protein, which involves an additional activity-dependent binding to Gβγ besides its constitutive binding. The activity-dependent binding interferes with the interaction of Gβγ to the GIRK channel leading to the inactivation of the channel and the termination of the ion flux. Interestingly, there are two additional mechanisms of fast desensitization of $GABA_B$-activated K^+ currents that are mediated by other proteins acting at the G-protein. Regulators of G-protein signaling (RGS) promote desensitization by binding to Gα and enhancing the GTPase activity (Chuang et al., 1998, Ross and Wilkie, 2000, Jeong and Ikeda, 2001, Bender et al., 2004, Mutneja et al., 2005). On the other hand, GPCR kinase 2 (GRK2) induces desensitization by binding to Gβγ similar to KCTD12 (Raveh et al., 2010, Turecek et al., 2014). The desensitization induced by GRK2 differs to the one induced by KCTD12 in its irreversibility. GRK2 scavenges free Gβγ from channels by binding to the "hot spot" region of Gβ, which sequesters the heterotrimeric G-protein and renders the desensitization irreversible (Raveh et al., 2010). In contrast, KCTD12 enables a fast and reversible desensitization by avoiding the "hot spot" and shielding the channel binding site of Gβγ but allowing the heterotrimeric G-protein to reassemble and to enter a new activation cycle.

Another important aspect of fast desensitization is its lack in receptor specificity as it is induced on the postreceptor level. Indeed, we show that KCTD12 promotes desensitization not only of $GABA_B$ receptors but also of adenosine and metabotropic glutamate receptors. However, native KCTD12 exclusively binds $GABA_B$ receptors making its desensitization highly specific for $GABA_B$ receptors.

A further observation in this study was the acceleration of $GABA_B$ receptor responses induced by KCTD12. Generally, the association between receptor and G-protein is slow and limited by diffusion. As KCTD12 binds the receptor as well as the G-protein, it pulls both proteins together. This dual binding

might therefore explain the accelerated receptor response since it should overcome the slow diffusion-limited association between receptor and G-protein.

In summary, KCTD12 may allow to regulate the influence of $GABA_B$ receptors on synaptic transmission as it precisely times the activation kinetics and the desensitization of $GABA_B$ receptor responses. KCTD12 might thereby also prevent excessive GIRK channel activity, which is a molecular phenotype in mouse models of epilepsy, Down syndrome or addiction (Best et al., 2007, Federici et al., 2009, Beenhakker and Huguenard, 2010, Best et al., 2012). Therefore, future directions could investigate the effect of KCTD12-mediated fine modulation of $GABA_B$ receptor-activated K^+ current responses on diseases in which $GABA_B$ receptors or GIRK channels play a key part. It would be interesting to study whether such reduced GIRK currents prevent seizures in absence epilepsy or counteract the increased GIRK activity found in a mouse model of Down syndrome.

The second part of this work describes the interplay between two mechanisms that regulate $GABA_B$ receptor responses. One of these mechanisms was previously described to decrease slow $GABA_B$ receptor desensitization by increasing $GABA_B$ receptor stability upon PKA phosphorylation of the $GABA_{B2}$ subunit at S892 (Couve et al., 2002, Fairfax et al., 2004). The other one is a much faster process that is induced by the auxiliary $GABA_B$ receptor subunit KCTD12, which leads to a rapid desensitization of $GABA_B$ receptor-activated K^+ currents. We show that S892 phosphorylation regulates not only the slow $GABA_B$ receptor desensitization but as well the fast type of receptor desensitization. S892 phosphorylation rearranges KCTD12 at the receptor thereby slowing KCTD12-induced desensitization. This observation was evident in heterologous cells as well as in hippocampal neurons, where knock-in mice with a S892 to alanine mutation show an accelerated K^+ current desensitization compared to wild-type mice. The assembly of KCTD12 with the receptor in turn increases tonic S892 phosphorylation promoting $GABA_B$ receptor stability in the cell surface. These results indicate that the mechanism of slow and fast desensitization influence each other.

Hippocampal $GABA_B$ receptors desensitize only moderately despite a high expression of KCTD12, which might be explained by the above described phosphorylation-dependency of $GABA_B$ receptor desensitization and a high tonic activity of PKA in the hippocampus (Couve et al., 2002). As $GABA_B$ receptors are negatively coupled to adenylyl cylase and thereby decrease PKA activity, repeated receptor activation sharpens the K^+ current response due to a feedback loop to its own phosphorylation. In addition to this homologous regulation of desensitization, other GPCRs that stimulate the cAMP/PKA signaling might regulate $GABA_B$ receptor desensitization in a heterologous manner. Furthermore, neuronal processes that affect PKA activity, like learning and memory, aging, seizures or NMDA-mediated synaptic plasticity (Jay et al., 1998, Vázquez et al., 2000, Thibault et al., 2001, Liu et al., 2010), may equally contribute to the regulation of $GABA_B$ receptor desensitization.

S892 might further be a potential binding site for interaction partners that bind or unbind dependent on its phosphorylation. Examples for such proteins are the multi-PDZ domain protein 1 (MUPP1) and 14-3-3 proteins (Fu et al., 2000, Parker et al., 2003, Balasubramanian et al., 2007, Jones et al., 2009). Binding of MUPP1 was shown to affect the signaling of 5-HT_{2C}Rs and $GABA_B$ receptors, whereas 14-3-3 proteins function as dimeric scaffolding proteins to enable the recruitment of different constituents into large

protein complexes. It is therefore possible that phosphorylation of S892 in $GABA_{B2}$ promotes the binding of such interaction partners to modulate the signaling of $GABA_B$ receptors.

The C-terminus of $GABA_{B2}$ is not only the platform for protein interactions but is also important for the activation of the G-protein (Margeta-Mitrovic et al., 2001, Schwenk et al., 2010, Turecek et al., 2014). As KCTD12-induced K^+ current desensitization is activity-dependent, a conformational rearrangement between the $GABA_B$ receptor and the G-protein upon S892 phosphorylation might reduce the rate of G-protein activation and thereby its kinetics. The physiological relevance of the phosphorylation-dependent regulation of $GABA_B$ receptor desensitization may be the modulation of slow inhibitory postsynaptic potentials (IPSPs). Synaptic activation of $GABA_B$ receptors in dendrites produces slow IPSPs that are carried by GIRK channels (Luscher and Slesinger, 2010). $GABA_B$ receptors and GIRK channels are primarily localized peri- or extrasynaptically (Lopez-Bendito et al., 2002, Kulik et al., 2003, Kulik et al., 2006) and the kinetic of slow IPSPs is predominantly determined by the diffusion of GABA into the extrasynaptic space and its reuptake by GABA transporters (Thompson and Gahwiler, 1992, Isaacson et al., 1993, Scanziani, 2000, Beenhakker and Huguenard, 2010). The duration of slow IPSPs is on the order of hundreds of milliseconds and even fast K^+ current desensitization in the presence of KCTD12 is unlikely to contribute significantly to the kinetic of individual slow IPSPs. However, activation of postsynaptic $GABA_B$ receptors usually requires the concerted action of multiple interneurons or repetitive stimulation (Dutar and Nicoll, 1988, Isaacson et al., 1993, Scanziani, 2000, Kulik et al., 2006) and it is therefore important to consider desensitization during sustained activation. Given that KCTD12-mediated desensitization persists for seconds (Turecek et al., 2014), it is expected that repetitive activation of $GABA_B$ receptors, as it is occurring during in vivo firing pattern, will produce slow IPSPs with a reduced amplitude. While further work is needed to investigate whether the temporal regulation of K^+ current desensitization by KCTD12 influences physiological processes, the present results provide the first evidence that this mechanism is controlled by second messenger kinases.

The third part investigates the allosteric influence of auxiliary KCTD subunits on pharmacological properties of $GABA_B$ receptors. KCTDs bind to both receptor and G-protein. Therefore, it is possible that they allosterically regulate the signaling of $GABA_B$ receptors. KCTDs as well as positive allosteric modulators (PAMs) bind to the $GABA_{B2}$ subunit making an interplay or interference very likely. KCTDs were shown to increase the potency of GABA (Schwenk et al., 2010). The PAM GS39783 increases both agonist binding-affinity and efficacy of the receptor response indicating a modulatory effect of GS39783 on both the orthosteric agonist binding-site and the G-protein activation (Urwyler et al., 2003, May et al., 2007). Here we show that both effects of GS39783 are independent on the presence of the KCTD proteins. In general, all KCTDs had almost no allosteric influence on G-protein activation or on the orthosteric agonist binding-site, which supports that the KCTDs mainly modulate the receptor response at the level of the G-protein. Furthermore, we found that $GABA_B$ receptor associated KCTD8 reduces the basal activation of G-proteins. This was observed in the [^{35}S]GTPγS binding assay as well as in BRET experiments and is possibly induced by a conformational rearrangement in the G-protein that interferes with the G-protein activation by the receptor. This study revealed the first functional difference between KCTD8 and KCTD16.

The fourth part focuses on new protein-protein interactions within the $GABA_B$ receptor complex and their potential to influence receptor signaling. This work revealed that the auxiliary $GABA_B$ receptor subunit KCTD16 interacts with two members of the 14-3-3 protein family, ε and γ. 14-3-3 proteins regulate the expression of KCTD16 by promoting lysosomal degradation (mediated by 14-3-3 γ) and enhancing the dimerization and stabilization of KCTD16 (mediated by 14-3-3 ε). Additionally, the $GABA_{B2}$ expression is modulated by 14-3-3 proteins when KCTD16 is co-expressed resulting in altered $GABA_B$ receptor signaling as observed in an assay that monitors PKA activity. 14-3-3 proteins furthermore seem to interfere with the interaction between KCTD16 and cullin3 (CUL3), another newly described interaction.

The auxiliary $GABA_B$ receptor subunits were first described in 2010 and the research to uncover their functions is still ongoing (Schwenk et al., 2010). Until now, the best described member of this family is KCTD12. In contrast to KCTD12, which was shown to drastically affect $GABA_B$ receptor signaling and G-protein activation, an important function for KCTD16 was not shown so far. The current study characterizes a protein-protein interaction specific for KCTD16 and gives a first impression about its importance for the signaling of $GABA_B$ receptors. KCTD16 binds 14-3-3 proteins with its H2-domain. These interactions are specific for the H2-domain of KCTD16 as KCTD8, which also contains an H2-domain, does not bind 14-3-3 proteins. Besides the antagonizing effect of the H2-domain on fast $GABA_B$-activated K^+ current desensitization, this domain was not assigned to any function yet. The result that 14-3-3 proteins bind this domain may indicate that it also offers additional binding sites for other proteins specific for KCTD16. Another finding, and difference to KCTD8, is the presence of a PEST sequence in the H2-domain of KCTD16. This sequence is described to promote rapid protein degradation. Although it is still under investigation whether this sequence is functional in KCTD16, it would explain the poor heterologous expression of KCTD16 and why its expression is improved by the deletion of the H2-domain. Future experiments will determine the half-life of KCTD16 and will compare the stability of wild-type KCTD16 to a KCTD16 mutant that lacks the PEST sequence. The observation that KCTD16 protein levels increase in the presence of proteasomal inhibitors support the hypothesis of a PEST-dependent degradation of KCTD16. However, co-expressing 14-3-3 γ redirected the degradation pathway for KCTD16 from the proteasome to the lysosome. Taking into account that cytosolic proteins are mainly degraded by the proteasome and membrane-bound proteins by the lysosome, we propose the following model. In the absence of 14-3-3 γ, KCTD16 is located in the cytosol and is rapidly degraded by the proteasome, which is initiated by the PEST-sequence. The binding of 14-3-3 γ to KCTD16 masks the PEST sequence and translocates KCTD16 to the membrane to facilitate $GABA_B$ receptor interaction. Once bound to the $GABA_B$ receptor, the whole receptor complex is degraded by the lysosome as we also see decreased $GABA_{B2}$ levels in the presence of 14-3-3 γ. The potential underlying mechanism will be discussed in the last paragraph of this chapter.

Whereas the above mentioned mechanism is defined for cell types or brain areas of high 14-3-3 γ expression, areas of high 14-3-3 ε expression show increased stability of both KCTD16 and $GABA_B$ receptors. 14-3-3 ε stabilizes KCTD16 by masking the PEST-sequence on one hand (as 14-3-3 γ) and by promoting the tetramerization of KCTD16 on the second hand. All KCTDs contain a tetramerization domain and were shown to bind the $GABA_B$ receptor in a ratio of 4:1, which confirms their ability to form tetramers (Schwenk et al., 2010). Nevertheless, it was never addressed whether KCTD proteins

exclusively form tetramers or whether their functionality depends on tetramer formation. The present study suggests that KCTD16 does not exist as monomers but forms higher-order oligomers. 14-3-3 ε further stabilizes the $GABA_B$ receptor itself (only when KCTD16 is co-expressed) by elevating the expression levels of $GABA_{B2}$. The following paragraph will also aim to address this mechanism.

The impact of the KCTD16/14-3-3 interaction on the signaling of $GABA_B$ receptors is at this stage still under investigation. First data already indicate that KCTD16 together with 14-3-3 proteins affects the expression of $GABA_B$ receptors and their signaling through Gα. This suggests a change in the number of functional $GABA_B$ receptors at the plasma membrane. Still, it is necessary to further underline this hypothesis by quantifying the amount of membrane-bound $GABA_B$ receptors by cell surface ELISA. The question arises how this regulation is achieved. We found that KCTD16 interacts with CUL3, a member of the cullin-RING ubiquitin ligase family. Other members of the KCTD family were already shown to interact with cullins providing them with an adaptor function for cullin-mediated ubiquitination and degradation (De Smaele et al., 2011). Therefore, it can be assumed that KCTD16 is an adaptor protein for CUL3 and $GABA_B$ receptors a substrate for cullin-mediated degradation. In this scenario, CUL3 would be recruited to $GABA_B$ receptors by KCTD16 to ubiquitinate the receptor. K800 in $GABA_{B2}$ was shown to be a putative ubiquitination site and might be the required target ubiquitination site (Na et al., 2012). As K63-linked polyubiquitination can be achieved by CUL3 (Jin et al., 2009, Han et al., 2010) and as it is the signal for lysosomal degradation, it would promote the degradation of the whole $GABA_B$/KCTD16 complex. The role of 14-3-3 proteins would be the following dependent on their isoform. 14-3-3 ε would interfere with the interaction between KCTD16 and CUL3 resulting in higher expression of $GABA_B$ receptors due to an inhibition of degradation. On the other hand, 14-3-3 γ would drive the lysosomal degradation of the $GABA_B$ receptor/KCTD16 complex as its binding to KCTD16 is not mutually exclusive with the binding of CUL3. To validate these hypotheses, it is necessary to show that CUL3 exists in a complex with $GABA_B$ receptors containing KCTD16 and that $GABA_{B2}$ K800 is K63-linked polyubiquitinated by CUL3. More experiments are also needed to underlay the finding that 14-3-3 ε indeed blocks the interaction between KCTD16 and CUL3 and to further characterize the binding between KCTD16 and CUL3.

Finally, it is important to note that $GABA_B$ receptors are not only modulated by other proteins, they can also regulate the signaling of their interaction partners. An ongoing project, in collaboration with the group of Jan Siemens, focuses on the modulation of TRPV1 sensitization by $GABA_B$ receptors. The capsaicin receptor TRPV1 is a nonselective cation channel that is mainly expressed in somatosensory, nociceptive neurons (Caterina et al., 1997). TRPV1s are sensitive to noxious stimuli and initiates protective reflexes to prevent tissue damage. Sensitization of TRPV1, which manifests in a low activation threshold of receptors, leads to hyperalgesia and pain hypersensitivity (Caterina et al., 2000). Antagonists that block TRPV1 have analgesic properties but concurrently produce hyperthermia (Julius, 2013). Drugs that prevent or counteract the sensitization of TRPV1 are therefore a more promising approach for pain therapy. Current data of this project reveal so far that activation of $GABA_B$ receptors inhibits TRPV1 sensitization to various inflammatory stimuli without affecting TRPV1 activity. The discovery of the underlying mechanism would be of high added value as inhibiting the sensitization of TRPV1s is esteemed to be one of the most promising approaches for pain therapy.

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6 CURRICULUM VITAE

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PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

Publications

- Rajalu M, Fritzius T, **Adelfinger L**, Jacquier V, Besseyrias V, Gassmann M and Bettler B; Pharmacological characterization of $GABA_B$ receptor subtypes assembled with auxiliary KCTD subunits; Neuropharmacology. 2014 Sep 6. pii: S0028-3908(14)00303-7.
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Scientific talks

- PhD Retreat Biozentrum, CH (’13)
- PhD Retreat Department of Biomedicine, CH (’13)

Poster presentation

- PhD Retreat Department of Biomedicine, CH (’14)
- Society for Neuroscience Meeting, US (’13)
- PhD Retreat Biozentrum, CH (’12)