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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.

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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.

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.

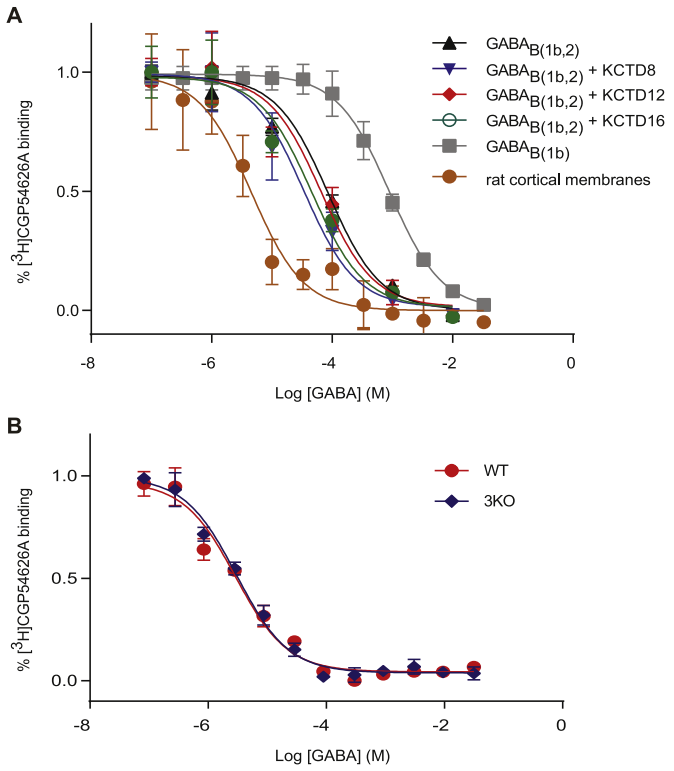

Fig. 1. Effects of the KCTDs on [^{3}H]CGP54626A displacement by GABA. (A) [^{3}H]CGP54626A displacement curves with membranes of HEK293T cells transiently transfected with either $GABA_{B(1b,2)}$ alone (black triangles, point up) or $GABA_{B(1b,2)}$ in combination with KCTD8 (blue triangles, point down), 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

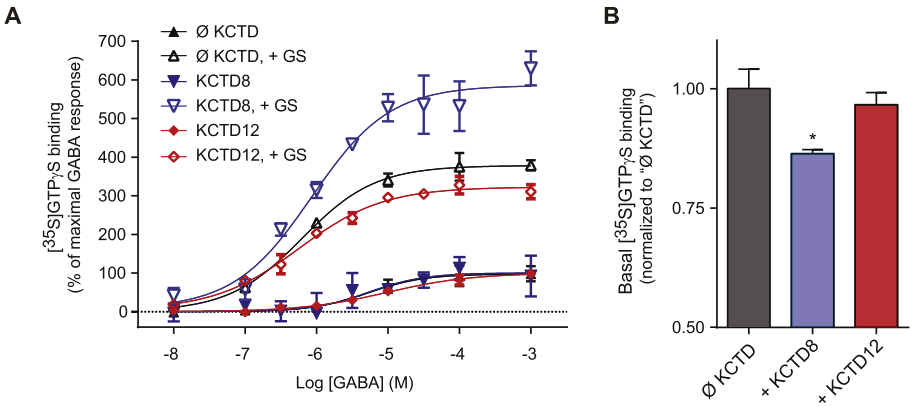

Fig. 2. Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in [^{35}S]GTPγS binding experiments. (A) GABA-induced [^{35}S]GTPγS binding to membranes of CHO-$GABA_B$ cells stably expressing either $GABA_{B(1b,2)}$ alone (ø KCTD, black triangles, point up) or $GABA_{B(1b,2)}$ in combination 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

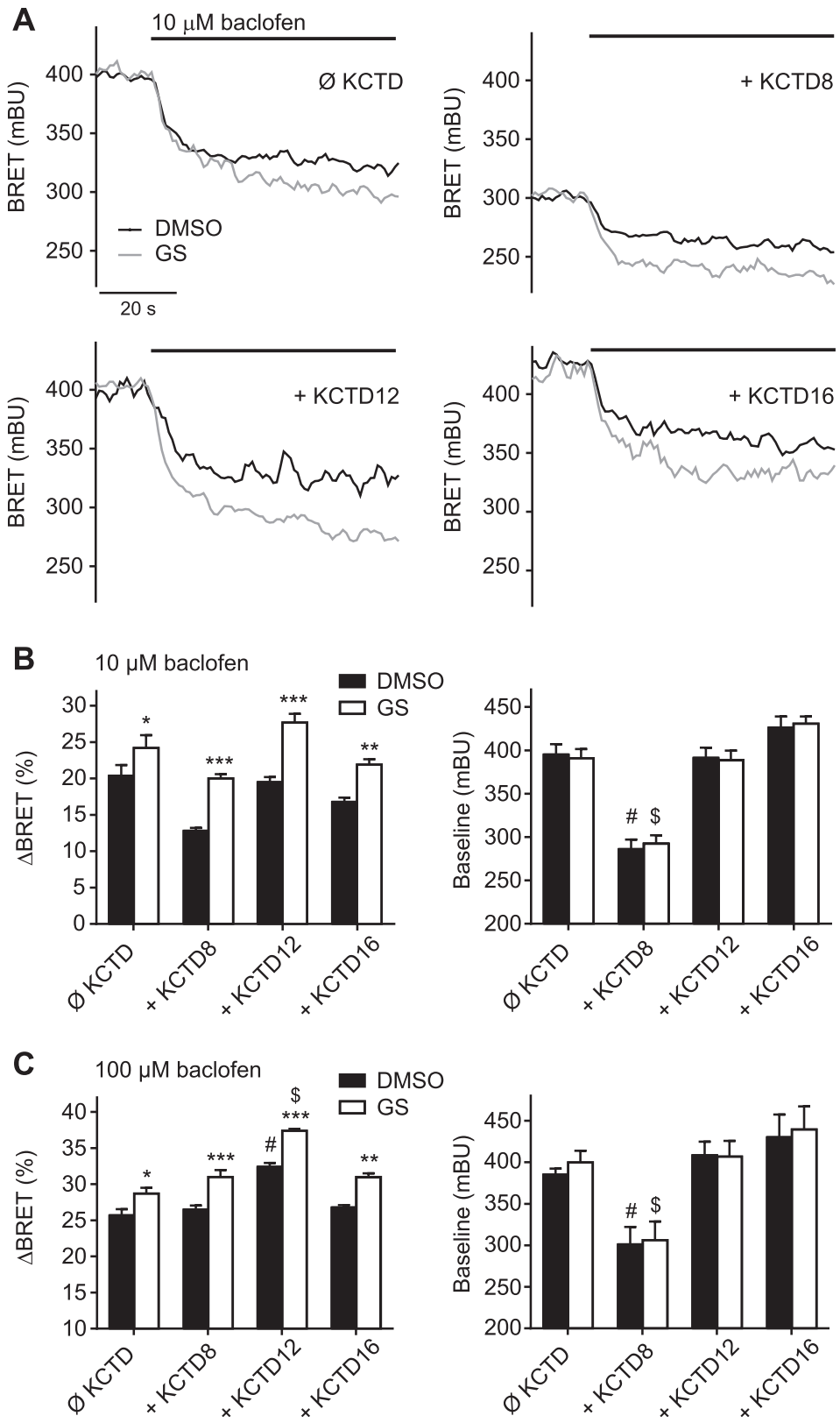

Fig. 3. Effects of the KCTDs on allosteric modulation of recombinant $GABA_BRs$ in BRET G-protein activation assays. (A) Representative baclofen-induced changes in the BRET signal (milli BRET units, mBU) with CHO-$GABA_B$ cells co-expressing Gαo-Rluc, Venus-Gγ2 and Gβ2 with or without KCTD8, KCTD12 or 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.

$GABA_BRs$ 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

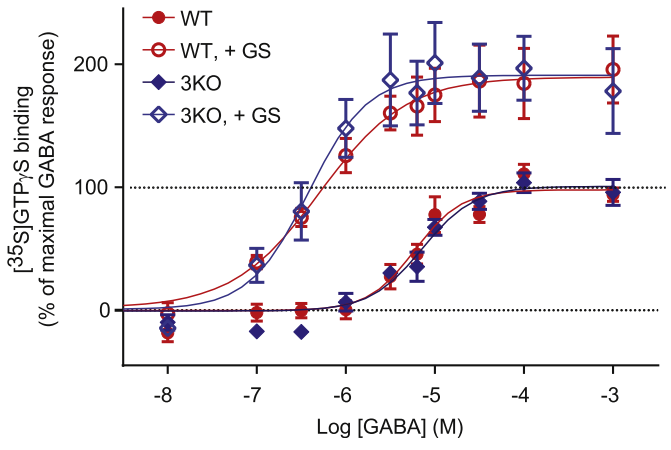

Fig. 4. Effects of the KCTDs on allosteric modulation of native $GABA_BRs$ in [^{35}S]GTPγS binding experiments. Effect of GS39783 on GABA-induced [^{35}S]GTPγS binding to brain membranes of wild-type (WT, red circles) or *Kctd8/12/16*$^{-/-}$ triple knockout mice (3KO, blue diamonds). Filled symbols 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 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

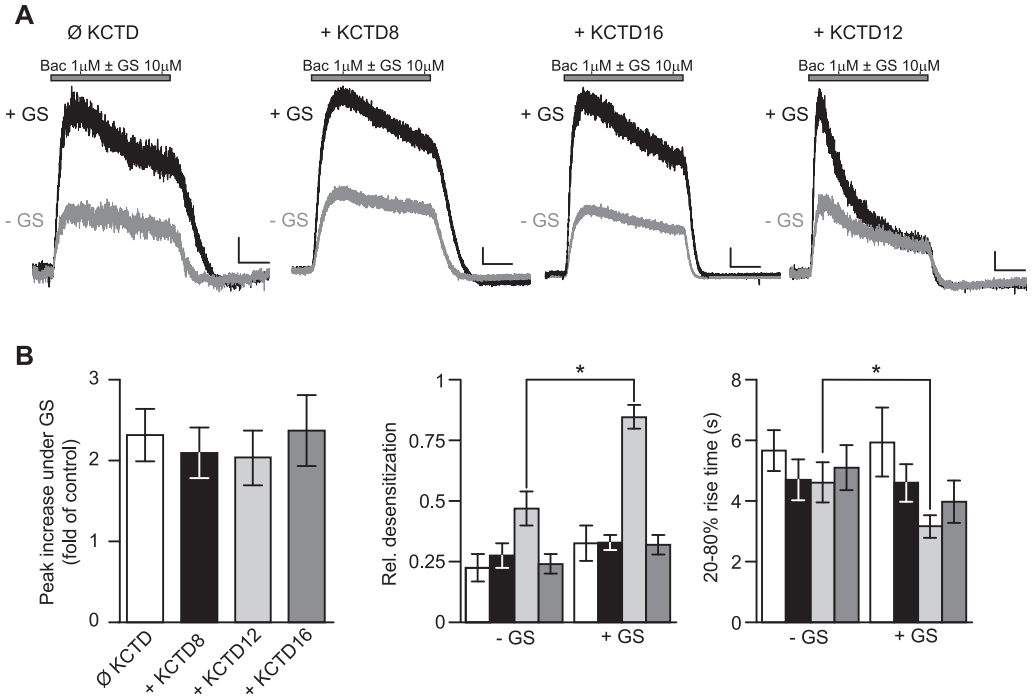

Fig. 5. Effects of the KCTDs on allosteric modulation of $GABA_BR$-activated Kir3 currents in transfected CHO cells. (A) Representative traces of $GABA_B$-activated Kir3 currents recorded at −50 mV from CHO-$GABA_B$ cells co-expressing Kir3 channels with or without KCTD proteins. Control traces evoked 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.

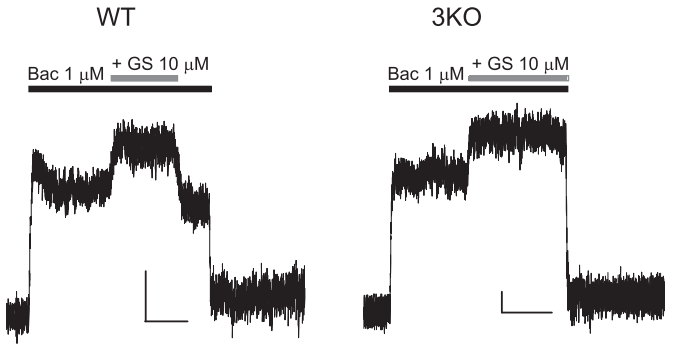

Fig. 6. Effects of the KCTDs on allosteric modulation of $GABA_BR$-activated K^+ currents in native neurons. Representative traces of $GABA_B$-activated Kir3 currents recorded at −50 mV from cultured hippocampal neurons of wild-type (WT, left) or *Kctd8/12/16*$^{-/-}$ triple knockout mice (3KO, right). 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

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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