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Auxiliary $GABA_B$ Receptor Subunits Uncouple G Protein $\beta\gamma$ Subunits from Effector Channels to Induce Desensitization

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SUMMARY

Activation of K^+ channels by the G protein $\beta\gamma$ 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 $G\beta\gamma$ subunits induces desensitization by uncoupling $G\beta\gamma$ 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 $G\beta\gamma$ 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 $G\alpha$, leading to dissociation of $G\alpha\cdot GTP$ from $G\beta\gamma$. Released $G\alpha\cdot GTP$ and $G\beta\gamma$ have independent capacities to regulate effectors such as enzymes and ion channels. $G\beta\gamma$ 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 $G\alpha$ (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

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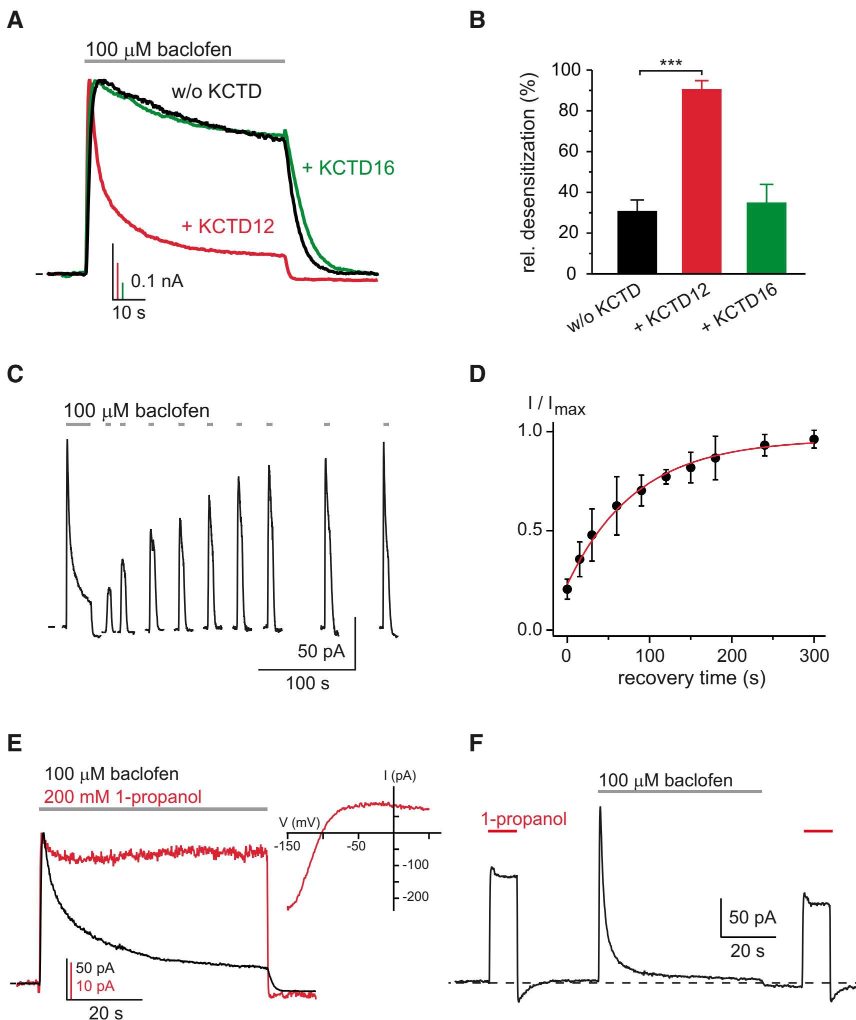

Figure 1. KCTD12-Induced Desensitization Is Activity Dependent, Reversible, and Operates Upstream of Kir3 Channels

(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

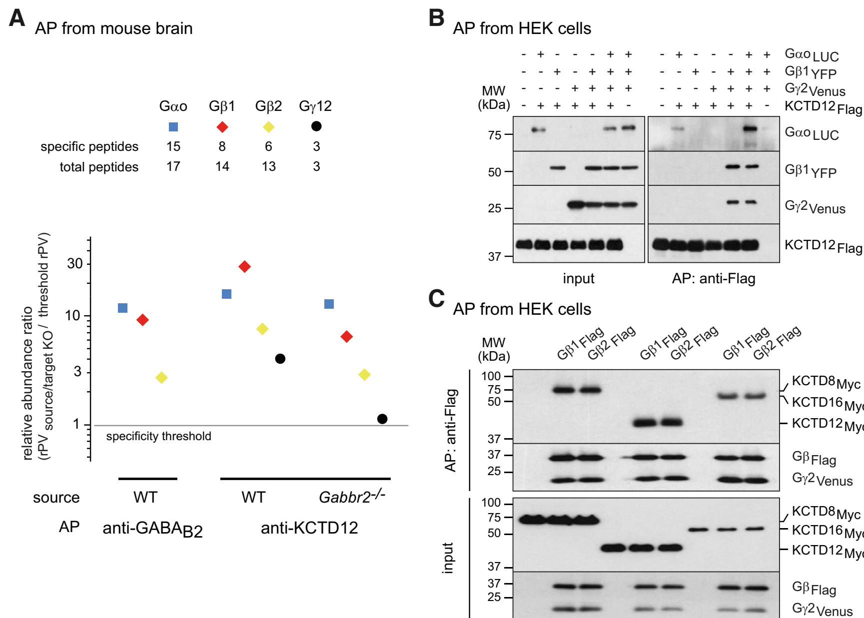

Figure 2. Binding of KCTDs to the G Protein Does Not Require $GABA_B$ Receptors

(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 ($Gβ1_{Flag}$) or Gβ2 ($Gβ2_{Flag}$). 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

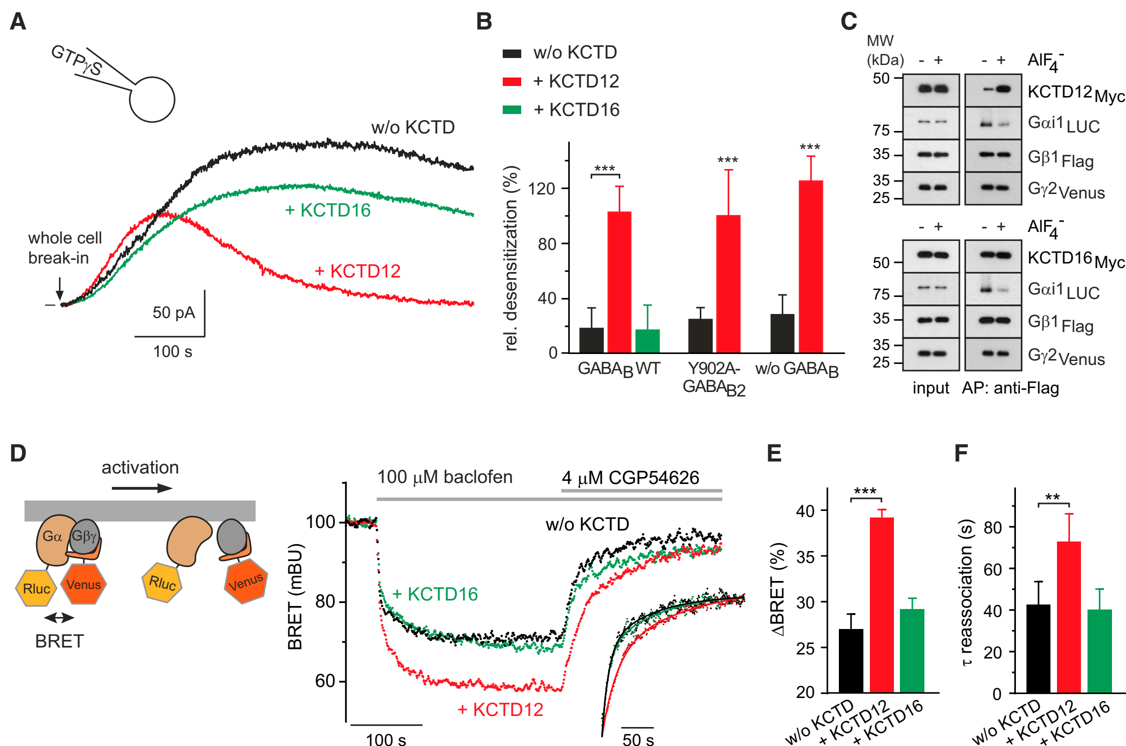

Figure 3. KCTD12-Induced Desensitization Requires Interaction with the Activated G Protein

(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 $G\alpha i1_{LUC}$, $G\beta 1_{Flag}$, $G\gamma 2_{Venus}$, 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

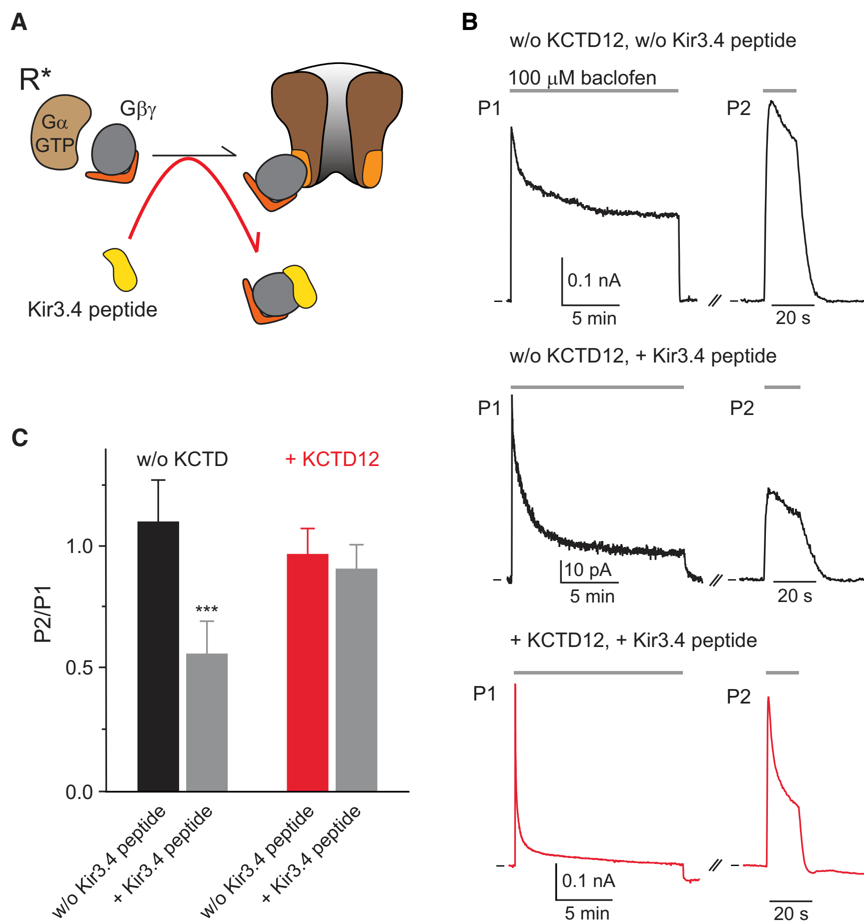

Figure 4. KCTD12 Occludes the Kir3 Interaction Site on $G\beta\gamma$

(A) Scheme illustrating activity-dependent interference with $G\beta\gamma$ 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 $G\beta\gamma$, consistent with an activity-dependent binding site on $G\beta\gamma$ 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 $G\beta\gamma$ 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 $G\beta\gamma$. Competition between KCTD12 and Kir3 channels for $G\beta\gamma$ reduces steady-state $G\beta\gamma$ interaction with the channels, which desensitizes the current response. Finally, the $G\alpha{\cdot}GDP$ subunit displaces KCTD12 from its activity-dependent binding site on $G\beta\gamma$ and reconstitutes the heterotrimeric G protein, which renders the desensitization mechanism reversible. To address whether KCTD12 occludes the channel binding site of $G\beta\gamma$ (Ford et al., 1998; Whorton and MacKinnon, 2013), we used a peptide derived from the $G\beta\gamma$ binding site of the Kir3.4 protein (Figure 4A). This Kir3.4-peptide inhibits $G\beta\gamma$ 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 $G\beta\gamma$. While allosteric effects of KCTD12 on Kir3.4-peptide binding cannot be fully ruled out, we consider this possibility less likely. The $G\beta\gamma$ 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;

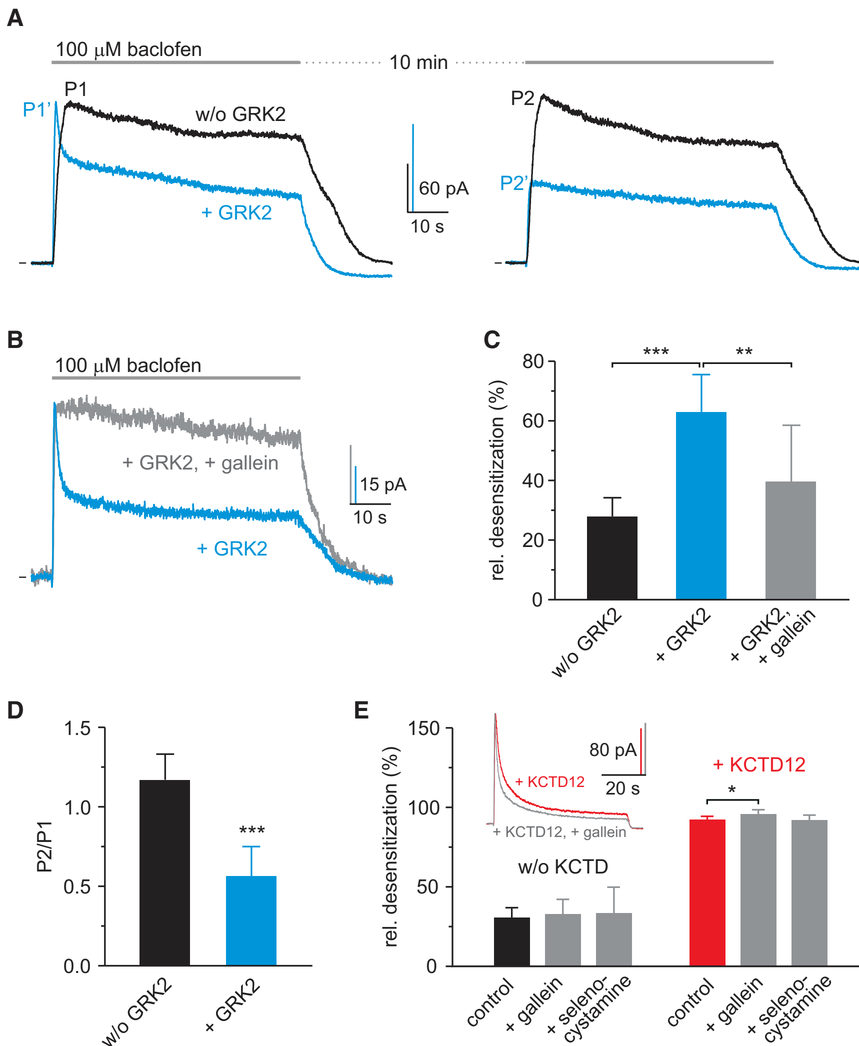

Figure 5. KCTD12 and GRK2 Differ in Their Binding to Gβγ and in the Reversibility of Desensitization

(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-$GABA_{B2}$

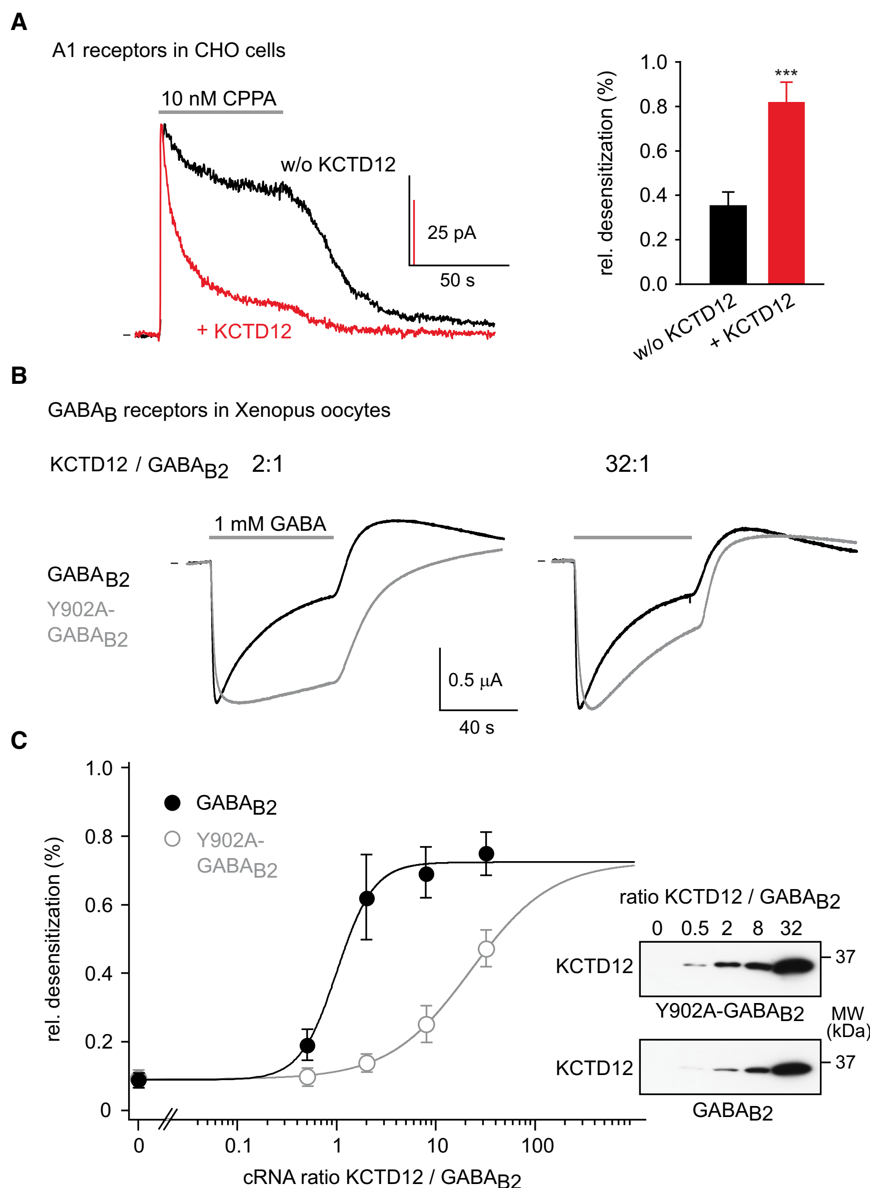

Figure 6. Receptor Specificity of KCTD12-Induced Desensitization Depends on the KCTD12/GABA$_B$ Ratio

(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).

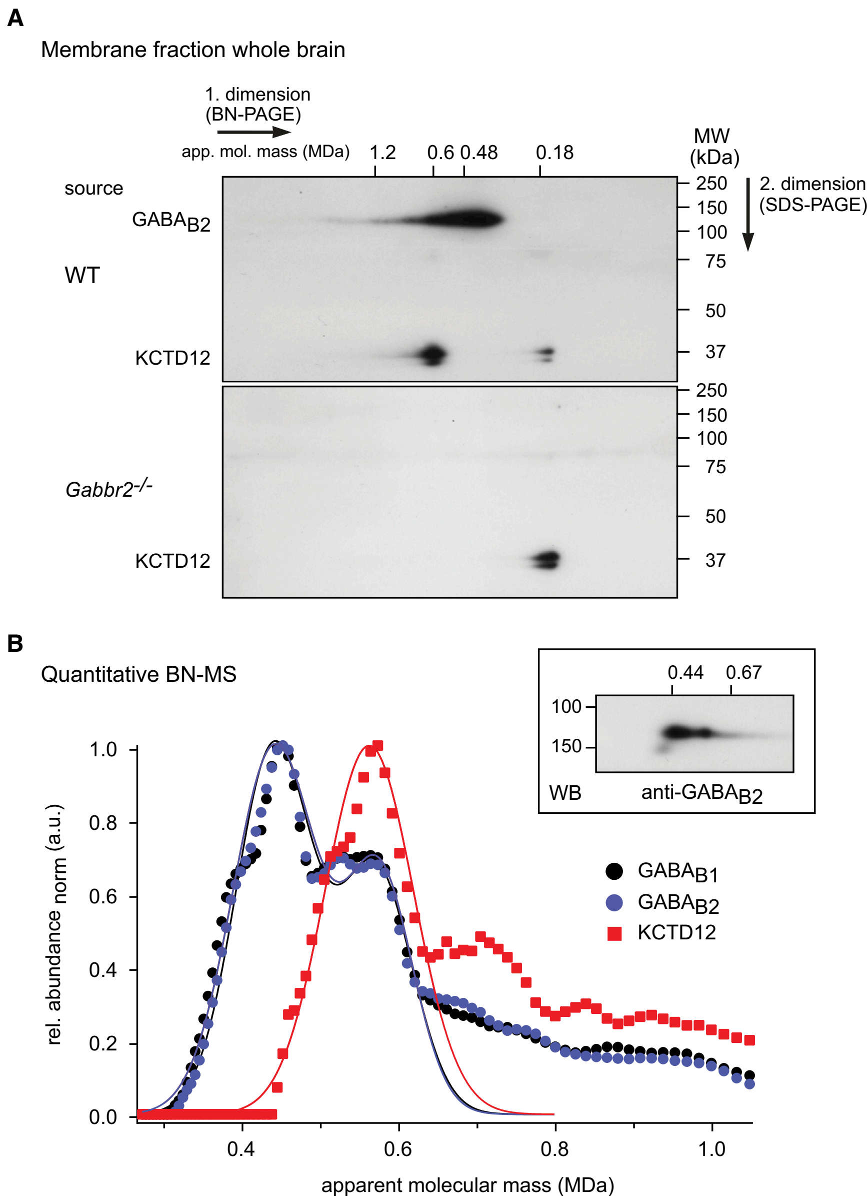

Figure 7. KCTD12 in the Adult Mouse Brain Is Exclusively Associated with $GABA_B$ Receptors

(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

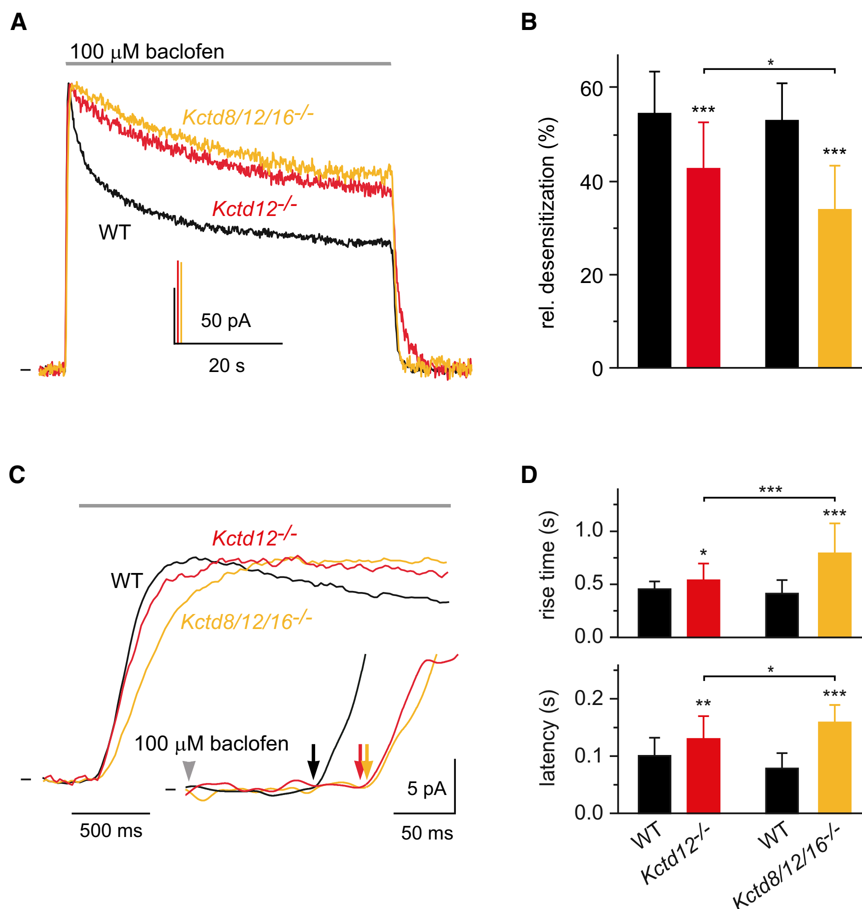

Figure 8. Altered $GABA_B$ Receptor-Activated K^+ Currents in *Kctd12*$^{-/-}$ and *Kctd8/12/16*$^{-/-}$ Mice

(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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