Characterization of a Novel Family of $GABA_B$ Receptor Interacting Proteins - KCTD8, 12, 12b, and 16

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Dekan

To my parents

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I. Summary

$GABA_B$ receptors are the G-protein coupled receptors for γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. They are heteromeric GPCRs which function as heterodimers composed of the $GABA_{B1}$ and $GABA_{B2}$ subunit. Presynaptic and postsynaptic $GABA_B$ receptors regulate Ca- and K-channels respectively via the $G_{\beta\gamma}$ subunits. Furthermore, $GABA_B$ receptors can activate and inhibit adenylyl cyclase through the $G_{\alpha i/o}$ and $G\beta\gamma$ subunits. Proteins that interact with $GABA_B$ receptors at the plasma membrane most likely contribute to the functional heterogeneity observed with native $GABA_B$ responses.

We identified members of the K^+ tetramerization domain containing (KCTD) protein family as novel $GABA_B$ receptor interacting proteins using immuno-affinity purification experiments from native mouse brain tissue. In separate affinity purification experiments, these proteins were also found to be associated with native Ca-channels. We have shown that native $GABA_B$ receptor exist as heteromultimers in a high molecular weight protein complex, tightly associated with KCTD8, 12, 12b, and 16.

KCTDs are able to form homo- and heterodimers, thus re-enforcing their role in complex formation with the $GABA_B$ receptor. We show that the KCTD-binding site is located within the intracellular C-term of the $GABA_{B2}$ subunit and that KCTDs interact with the receptor via their conserved N-termini. In addition, coassembly with KCTDs leads to alterations of $GABA_B$ receptor properties, such as an increase in agonist potency and alterations in G-protein signaling. When compared, the spatial distribution and the functional properties of KCTD8, 12, 12b, and 16 suggest overlapping but also distinct properties in the context of $GABA_B$ receptor function. In summary, this dissertation reveals KCTD8, 12, 12b, and 16 as important players within the $GABA_B$ receptor signaling complex and designates them as novel auxiliary subunits of the $GABA_B$ receptor.

II. Abbreviations

AMPK	5'AMP-dependent protein kinase
AP-2	activator protein-2
ARF	ADP-ribosylation factor
ATF4	activating transcription factor 4
BRET	bioluminescence energy transfer
BTB	Bric-a-brac, Tramtrack, Broad-complex
Ca^{2+}/CaM	Ca^{2+}/Calmodulin
CaMKII	Ca^{2+}/Calmodulin-dependent kinase II
cAMP	cyclic adenosine monophosphate
Cav	voltage-gated Ca^{2+} channel
C/EBP	CCAAT/enhancer-binding protein
CHOP	C/EBP homologous protein
CNS	central nervous system
COPI	coat protein I
CREB2	cAMP response element binding-protein 2
DIV	day in vitro
eGFP	enhanced green fluorescent protein
EPSC	excitatory postsynaptic current
ER	endoplasmic reticulum
ESI-MS/MS	electrospray-ionisation mass spectrometry
FRET	fluorescence energy transfer
GABA	gamma-amino butyric acid
$GABA_A$	gamma-amino butyric acid type A
$GABA_B$	gamma-amino butyric acid type B
$GABA_{B1}$-/-	$GABA_{B1}$ deficient
$GABA_{B2}$-/-	$GABA_{B2}$ deficient
$GABA_C$	gamma-amino butyric acid type C
$GABA_BR$	$GABA_B$ receptor
GAP	GTPase activating proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GHB	gamma-hydroxybutyrate
GPCR	G-protein coupled receptor
GTP	guanosine-5'-triphosphate

HNK-1	human natural killer-1
IPSC	inhibitory postsynaptic current
KCTD	K^+ channel tetramerization domain containing
Kv	voltage-gated K^+ channel
LTD	long term depression
LTP	long term potentiation
mGluR	metabotropic glutamate receptor
VFTM	venus flytrap module
P	postnatal day
PCNA	proliferating cell nuclear antigen
PDIP1	Polymerase delta-interacting protein 1
PDZ	PSD-95/Discs-large/ZO-1
POZ	Pox virus and Zinc finger
PTB	phosphpotyrosine binding
Shh	sonic hedgehog homolog
T1	tetramerization domain
7TM	seven transmembrane
TGN	trans-Golgi network
TNF-α	Tumor necrosis factor α
TNFAIP1	Tumor necrosis factor, alpha-induced protein 1
Y2H	yeast-two-hybrid

III. Preface

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

Native $GABA_B$ receptors are heteromultimers with a family of auxiliary subunits

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(submitted)

The C-terminus of KCTD12 promotes desensitization of $GABA_B$ receptors in neurons

Jim Y. Tiao*, Riad Seddik*, Michaela Metz, Bolette Christiansen, Valerie Besseryias, Thomas Oertner, Bernhard Bettler

(in preparation)

Differential expression pattern of the novel auxiliary subunits of the $GABA_B$ receptor - KCTD8, 12, and 16

Michaela Metz, Nicole Schaeren-Wiemers and Bernhard Bettler

(in preparation)

1 Introduction

GABA receptors

γ-Aminobutyric acid (GABA), first identified by Robert and Frankel, is the most widely distributed inhibitory neurotransmitter in the central nervous system (Roberts and Frankel, 1950). After GABA is released from presynaptic nerve terminals under the regulation of intracellular Ca^{2+}, its actions are mediated by two main classes of receptors, ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors.

Activation of $GABA_A$ receptors leads to an increase in Cl^- conductance, whereas $GABA_B$ receptor activation produces G-protein-mediated responses. Activation of all receptor subtypes induces a hyperpolarization of the cell that forms the basis of the inhibitory action of GABA.

The $GABA_A$ receptors belong to the superfamily of ligand-gated ion channels that includes the nicotinic acetylcholine, strychnine-sensitive glycine, and 5-HT_3 serotonin receptors. They are assembled from a large family of subunit genes (α1-6, β1-4, γ1-4, δ, ε, π, θ, ρ1-3) and form heteromeric chloride channels. Five subunits can combine in different permutations to form $GABA_A$ channels that give rise to a large variety of $GABA_A$ receptors with distinct pharmacological properties; 60% of $GABA_A$ receptors in the brain are composed out of two α1, two β2, and one γ2 subunit (Mohler, 2006). Ligands acting on the $GABA_A$ receptor are commonly used as therapeutics, e.g. allosteric modulators like benzodiazepines and barbiturates, which exhibit anxiolytic, antiepileptic, muscle relaxant, and hypnotic effects.

The ρ-subunits do not assemble with α- or β- subunits, but form homo- and heteromeric channels among themselves. These receptors are therefore sometimes designated as a separate class of ionotropic GABA receptors, termed $GABA_C$ receptors (Chebib, 2004). They exhibit unique pharmacological properties such as being sensitive to cis-4-aminocrotonic acid, but insensitive to bicuculline and benzodiazepines. $GABA_C$ receptors are mainly found in the retina. Controversy remains, however, whether these receptors constitute an independent class of receptors or if they are a subtype of $GABA_A$ receptors.

$GABA_B$ receptors belong to the class C (metabotropic glutamate/pheromone) of G protein-coupled receptors (GPCRs). They are seven transmembrane (7TM) receptors that activate second messenger systems through G-protein coupling. G protein-coupled receptors constitute by far the largest family of cell surface proteins involved in signaling across biological membranes with approximately 950 genes in the human genome encoding proteins belonging to this superfamily (Takeda et al., 2002). GPCRs modulate a wide range

of physiological processes. They are implicated in numerous diseases and form the largest class of therapeutic targets.

The $GABA_B$ receptor

History

$GABA_B$ receptors were first described by Bowery et al. in the late seventies. Researchers had observed that application of bicuculline, the first specific GABA receptor antagonist, only partially inhibited the effects elicited by GABA. This led to the conclusion that there must exist different, but foremost, bicuculline insensitive subtypes of GABA receptors later followed by the description of the binding site (Bowery et al., 1979; Bowery et al., 1981; Hill and Bowery, 1981). The first evidence that the $GABA_B$ receptor is a G-protein coupled receptor came from the observation that application of guanyl nucleotides reduces agonist affinity of $GABA_B$ receptors (Hill et al., 1984; Kerr and Ong, 1995). The development of $GABA_B$ receptor specific agonists and antagonists, especially the high-affinity $GABA_B$ radioligand antagonists, such as ^{125}I-CGP64213 made expression cloning using a binding assay possible. It allowed the isolation of $GABA_{B1a}$, and $GABA_{B1b}$ cDNAs (Kaupmann et al., 1997), and shortly after the $GABA_{B2}$ cDNA (Jones et al., 1998; Kaupmann et al., 1998). Molecular cloning of the $GABA_B$ receptors became eminently important as it challenged the traditional dogma of GPCR activation and the subsequent mechanisms of G-protein mediated signaling. GPCRs were traditionally conceptualized as monomeric proteins until $GABA_B$ receptors were shown to require heteromerization to form a functional receptor (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Dimerization of GPCRs was at the time a novel concept and triggered a large body of work resulting in today's commonly accepted view of functional dimerization for many GPCRs (Fan and Hendrickson, 2005; Fotiadis et al., 2004). The $GABA_B$ receptor is perhaps the best example demonstrating the functional importance of oligomerization as the heterodimerization of $GABA_{B1}$ and $GABA_{B2}$ subunits is a prerequisite for the trafficking of the receptor to the cell surface, and the allosteric interactions between the two $GABA_B$ receptor subunits are critical for agonist-induced activation. These findings together with evidence from other GPCRs existing as oligomeric clusters have been and still are conducive to the changing perception of GPCR oligomerization and its significance in GPCR signaling.

Neurological disorders and therapeutic agents

Neurological disorders such as bipolar disorders, anxiety, epilepsy and depression often result from an imbalance between excitation and inhibition. Therefore $GABA_B$ receptors are

interesting drug targets. Although Baclofen (β-chlorophenyl-GABA, Lioresal®), the prototype $GABA_B$ receptor agonist, was introduced into therapeutic use already in 1972, this is still the only specifically $GABA_B$ targeting compound on the market. The muscle relaxant and anti-nociceptive actions of baclofen make it to the drug of choice in spasticity associated with cerebral palsy, multiple sclerosis, stiff-man syndrome, tetanus and for reduction of central pain, as occurs in stroke.

Recent preclinical and clinical studies have found that baclofen suppresses symptoms of alcohol withdrawal syndrome and effectively prevents relapse due to its ability to reduce alcohol intake and craving in alcoholic patients, which suggests baclofen as a promising pharmacological compound for the treatment of alcohol dependence (Addolorato et al., 2006). However, baclofen treatment leads to adverse reactions such as drowsiness, nausea, muscle weakness, hallucinations and mental confusion. Out of numerous synthesized compounds specifically targeting $GABA_B$ receptors (Bowery et al., 2002), only CGP 36724, a $GABA_B$ receptor antagonist is currently in clinical trials for treatment of mild cognitive impairment (Froestl et al., 2004). $GABA_B$ receptor antagonists have also been shown in several animal models to have antidepressant-like effects (Cryan and Kaupmann, 2005; Nowak et al., 2006).

Structure and Function

Members of Class C GPCRs, which include the metabotropic glutamate, calcium-sensing, vomeronasal, taste, and a family of orphan receptors, are characterized by a long extracellular ligand binding domain. The N-terminal domain of $GABA_B$ receptors contains a venus flytrap module (VFTM) harboring the GABA binding site, that was extensively described after homology modeling based on X-ray structures of bacterial periplasmic binding domains (Galvez et al., 1999; Galvez et al., 2000).

$GABA_B$ receptors exist as obligate heterodimers, composed of a $GABA_{B1}$ and a $GABA_{B2}$ subunit. Though similar in structure, only the $GABA_{B1}$ subunit binds the agonist. Dimerization with $GABA_{B2}$, however, allosterically enhances agonist binding (Galvez et al., 2001; Liu et al., 2004).

The two $GABA_B$ subunits assemble via their transmembrane domains (Pagano et al., 2001) and interaction of their intracellular coiled-coil domains (Kuner et al., 1999). Upon agonist binding to $GABA_{B1}$, conformational changes in the relative positions of the two VFTMs and heptahelical domains lead to G protein activation. G proteins bind to the third intracellular loop of the heptahelical domain of $GABA_{B2}$ (Havlickova et al., 2002) and convey signals to the different effectors of $GABA_B$ action. As $GABA_{B1}$ alone is retained in the endoplasmatic

reticulum (ER), the $GABA_{B2}$ subunit is also required for surface trafficking of the functional heteromeric receptor (Pagano et al., 2001). An RSRR ER retention signal located next to the coiled-coil region of $GABA_{B1}$ is responsible for intracellular retention. Heterodimerization with $GABA_{B2}$ shields the ER retention signal and ensures that only correctly assembled $GABA_B$ receptors are trafficked to the cell surface (Pagano et al., 2001). This is also evident in $GABA_{B2}$ knockout mice, whereby $GABA_{B1}$ is largely retained in the ER (Gassmann et al., 2004).

$GABA_B$ receptors couple predominantly to $G_{i\alpha}$- and $G_{o\alpha}$-type G proteins. Following G protein activation, Ca^{2+} channels are inhibited and K^+ channels activated by the $\beta\gamma$ subunit, whereas the α subunit influences adenylate cyclase activity and thus changes the level of the second messenger cyclic adenosine monophosphate (cAMP). $GABA_B$ receptors modulate synaptic transmission through pre- and postsynaptic actions. At presynaptic nerve terminals, activation of $GABA_B$ receptors leads to inhibition of voltage-gated N-type (Cav2.2) and P/Q-type (Cav2.1) Ca^{2+} channels, and thus decreases transmitter release. Presynaptic $GABA_B$ receptors act either as autoreceptors, which decrease the release of GABA or as heteroreceptors, which decrease the release of glutamate or other neurotransmitters. Postsynaptically $GABA_B$ receptors induce the activation of G-protein-activated inwardly rectifying K^+ (GIRK/Kir3) channels, which leads to an increased efflux of K^+ resulting in a slow hyperpolarization of the postsynaptic membrane. $GABA_B$ receptors induce a slow inhibitory postsynaptic current (IPSC) which can be distinguished from the fast IPSC mediated by $GABA_A$ receptors (Otis et al., 1993).

In addition, $GABA_B$ receptors inhibit adenylate cyclases types I, III, V, and VI through the activation of Giα- and Goα proteins which subsequently reduce cAMP levels. In crosstalks with Gs-coupled receptors, $GABA_B$ receptors can also increase the activity of adenylate cyclase types II, IV, and VII (Bowery et al., 2002). Among the effects of altered cAMP levels are the facilitation of vesicle priming (Sakaba and Neher, 2003) or the modulation of cAMP-dependent kinase, also known as protein kinase A (PKA), targeted K^+ channels (Gerber et al., 1993).

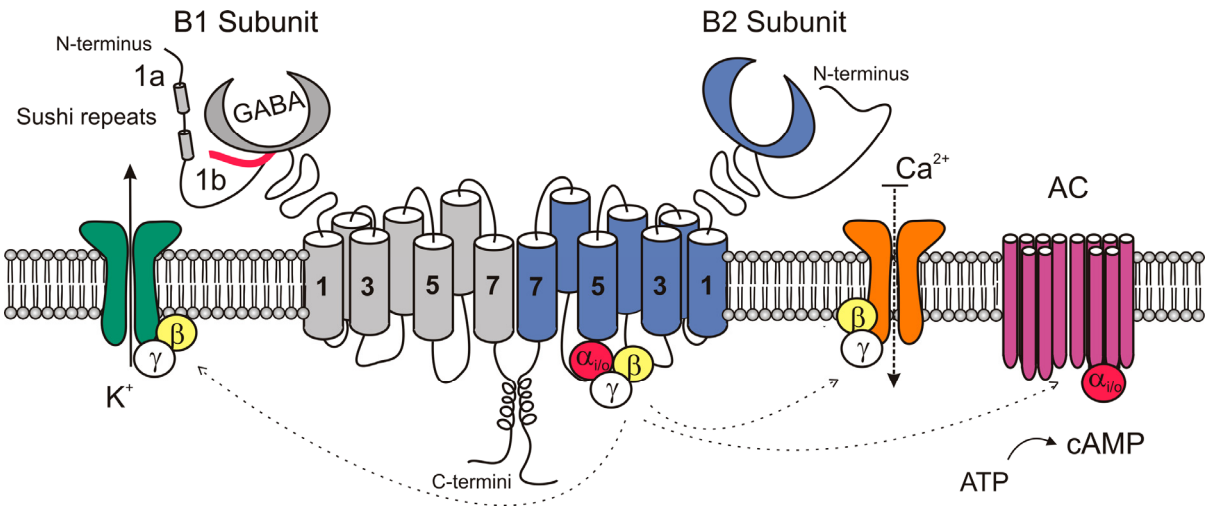

Figure 1-1: Structure and physiological role of $GABA_B$ receptors. $GABA_B$ receptors form obligate heterodimers composed of $GABA_{B1}$ and $GABA_{B2}$ subunits. The $GABA_{B1}$ subunit contains the GABA binding site whereas the $GABA_{B2}$ subunit couples to the G-protein. Two isoforms, $GABA_{B1a}$ and $GABA_{B1b}$, exist, differing in the two sushi domains at the N-terminus. Upon activation of $GABA_B$ receptors and the subsequent dissociation of the G-protein, the $G_{\alpha i/o}$ subunit inhibits adenylyl cyclase, whereas the $G_{\beta\gamma}$-dimer translocates and binds to effector channels. Presynaptically, $GABA_B$ receptor activation results in inhibition of voltage gated Ca-channels and therefore in the inhibition of neurotransmitter release. Postsynaptically, the coupling of the $G_{\beta\gamma}$-dimer to Kir3-type K–channels results in accelerated K^+ efflux and therefore in hyperpolarization of the postsynaptic cell.

Distribution and Localization

$GABA_B$ receptors are widely distributed in the central nervous system. They are highly expressed in the brain and spinal cord, but also present in other tissues. $GABA_B$ receptors are expressed in almost all neuronal populations in the brain, and have also been detected in nonneuronal cells (Charles et al., 2003). Amongst the brain regions, hippocampus, thalamus, cortex, and cerebellum coexpress the highest amounts of $GABA_{B1}$ and $GABA_{B2}$ subunits (Durkin et al., 1999; Fritschy et al., 1999; Margeta-Mitrovic et al., 1999).

Detailed studies in the hippocampus have revealed their subcellular localization. Presynaptically, the receptors have been detected at extrasynaptic sites, occasionally at glutamatergic terminals and, to a lesser extent, at GABAergic terminals. Postsynaptically $GABA_B$ receptors are found in dendritic shafts of principal neurons and interneurons, and in dendritic spines. The receptors are concentrated around glutamatergic synapses in spines but are evenly distributed over dendritic shafts of principal neurons contacted by GABAergic terminals (Kulik et al., 2003). $GABA_B$ receptors, therefore, are well positioned to modulate GABAergic and, especially, glutamatergic synapses.

Another peculiarity of $GABA_B$ receptors is their presence at extrasynaptic sites where they are activated by GABA spillovers originating from interneurons. Thereby $GABA_B$ receptor-mediated inhibition can be specifically enhanced during periods of intense neuronal activity.

GABA “spillover” occurs in cases of hyperexcitation accompanying, for example, epileptic seizures or during synchronous firing of neuronal networks. Simultaneous release of GABA from several interneurons, as appears during rhythmic hippocampal activity, was described by measurements from pairs of interneurons and pyramidal neurons in cultured hippocampal slices, and after blockade of GABA uptake. This results in the activation of postsynaptic $GABA_B$ receptors, which in turn control the oscillation frequency of the network (Scanziani, 2000). Based on these studies, therefore, mechanisms influencing the concentration of GABA outside the synaptic cleft can affect $GABA_B$ receptor activation.

Heterogeneity

Physiological and pharmacological studies have observed a large variability in the actions of native $GABA_B$ receptors, particularly regarding ligand binding, responses to agonists and antagonists, G-protein coupling and alterations of signal transduction pathway (Bonanno et al., 1997; Bonanno and Raiteri, 1993; Cruz et al., 2004; Cunningham and Enna, 1996). This suggests a multitude of $GABA_B$ receptor subtypes as observed in other GPCRs. Research in the $GABA_B$ receptor field was, for many years, greatly motivated by the aim of finding new splice variants that could encode differences observed for agonist affinity, effector channel conductance, or G-protein coupling. Surprisingly, none of the identified alternatively spliced $GABA_B$ subunit mRNAs have so far been demonstrated to participate in native $GABA_B$ receptor function. Genetic studies with $GABA_{B1}$ and $GABA_{B2}$ knockout mice show a complete absence of typical biochemical, electrophysiological and behavioral $GABA_B$ responses, supporting the idea that the $GABA_{B(1,2)}$ heterodimer provides all native $GABA_B$ functions.

The only receptor divergence which could be conclusively demonstrated comprises two isoforms of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$. These two isoforms are evolutionary conserved and arise from alternative transcriptional start site selection on the $GABA_{B1}$ gene. At the extracellular N-terminus, $GABA_{B1a}$, in contrast to $GABA_{B1b}$, contains two sushi domains, which are known protein-protein interaction sites. $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ are pharmacologically not distinguishable, however $GABA_{B1a}$ and $GABA_{B1b}$ subunits show different spatial and temporal expression patterns in the brain (Bischoff et al., 1999; Kaupmann et al., 1997). During early postnatal development, $GABA_{B1a}$ exceeds the $GABA_{B1b}$ isoform before it is downregulated around postnatal day 10 (P10). In parallel, $GABA_{B1b}$ is upregulated and reaches maximal expression levels at P10-20. $GABA_{B1b}$ is predominant during adulthood and forms the main receptor subtype $GABA_{B(1b,2)}$. Due to the lack of subtype-specific antibodies, it was only recently shown, through the use of mice selectively expressing $GABA_{B1a}$ or $GABA_{B1b}$ that the $GABA_{B1a}$ subunit isoform preferentially localizes to glutamatergic terminals, whereas $GABA_{B1b}$ is predominantly found on the postsynaptic side

(Vigot et al., 2006). Moreover, Vigot et al. showed that the differential subcellular localization of receptor subtypes also results in different functions. Whereas $GABA_{B(1b,2)}$ receptors predominantly mediate postsynaptic inhibition, $GABA_{B1a}$ presynaptically inhibits glutamate release (Guetg et al., 2009; Vigot et al., 2006). In this context it is easy to anticipate that the sushi repeats specifically traffic $GABA_{B1a}$ to or anchor $GABA_{B1a}$ at axonal sites via for example, interaction with proteins of the neuronal targeting machinery. Blein et al. reported an interaction with the extracellular matrix protein fibulin-2, but whether this contributes to $GABA_B$ receptor localization is unknown (Blein et al., 2004). More interestingly, our group recently described a secreted $GABA_{B1}$ subunit isoforms, $GABA_{B1j}$, which comprises the sushi domains, present in $GABA_{B1a}$ and showed that soluble sushi domains inhibit $GABA_B$ heteroreceptor function at glutamatergic terminals (Tiao et al., 2008).

Interacting molecules

As the receptor subtypes $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ were pharmacologically not distinguishable, studies have focused on the identification of interacting molecules that could account for the heterogeneity observed in native $GABA_B$ receptor function.

Extracellular interactions

Two extracellular binding partners of the $GABA_B$ receptor have been described. First, the HNK-1 (human natural killer-1) carbohydrate was proposed to be part of the underlying mechanism of perisomatic inhibition in the hippocampus. HNK-1 carbohydrate binds to $GABA_B$ receptors and is carried by many extracellular matrix proteins, among them tenascin-C and tenascin-R. HNK-1 mediated $GABA_B$ receptor activation in CA1 pyramidal cells, followed by K^+ outflux led to an inhibition of GABA release at perisomatic inhibitory synapses (Saghatelyan et al., 2003). Interestingly, HNK-1 is expressed in the cerebellum only in the posterior zone in Zebrin II-immunopositive stripes (Marzban et al., 2004) which partially overlaps with $GABA_B$ receptor localization (Fritschy et al., 1999). Second, the extracellular matrix protein fibulin-2 was found to interact with the N-terminal sushi repeats of $GABA_{B1a}$ (Blein et al., 2004). The sushi domains exhibit the only obvious difference between the $GABA_B$ receptor subtypes and were therefore strongly suggested to be involved in differential targeting of the $GABA_{B1}$ subunit, most likely through specific binding partners, like fibulin-2. However, fibulin-2 does not affect $GABA_B$ heteroreceptor function (Tiao et al., 2008).

A number of interacting proteins were identified in yeast-two-hybrid (Y2H) screens using the intracellular termini of $GABA_{B1}$ and $GABA_{B2}$ as bait. Most of them were suggested to be implicated in $GABA_B$ receptor trafficking and cell surface expression.

Coiled-coil domain interactions

Two members of the 14-3-3 family of proteins, 14-3-3η and 14-3-3ζ (Couve et al., 2001) bind to part of the coiled-coil domain and the ER retention signal of $GABA_{B1}$. Retention of $GABA_{B1}$ in the ER was shown to be mediated through the coat protein I complex (COPI) that mediates retrieval from the cis-Golgi to the ER in COPI-coated vesicles. Competition for $GABA_{B1}$ binding was reported between 14-3-3 proteins and COPI, however, only COPI seems to be important for surface trafficking of correctly assembled $GABA_B$ receptor heterodimers (Brock et al., 2005).

Some nuclear proteins were also reported to bind the coiled-coil domain of the $GABA_B$ receptor subunits, such as the activating transcription factor 4 (ATF4) and cAMP response element binding-protein 2 (CREB2). Upon activation of the $GABA_B$ receptor, researchers observed a translocation of ATF4/CREB2 either into or out of the nucleus (Vernon et al., 2001; White et al., 2000). The functional significance, however, remains to be clarified. In addition, $GABA_B$ receptor activation leads to an increased expression of an ATF4-responsive reporter gene (White et al., 2000). Interestingly, ATF4 has been found to differentially regulate $GABA_{B1a}$ and $GABA_{B1b}$ promoter activity (Steiger et al., 2004), which could account for the different expression patterns of the two isoforms. Another transcription factor, CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) has been shown to bind specifically to the $GABA_{B(1a,2)}$ subtype and to accumulate $GABA_{B(1a,2)}$ in the ER (Sauter et al., 2005). CHOP and $GABA_{B2}$ interact via their coiled-coil domains and in addition, the N-terminal domain of CHOP binds to a yet unidentified intracellular part in the $GABA_{B1a}$ subunit. The fact that the intracellular domains of $GABA_{B1a}$ and $GABA_{B1b}$ are identical, argues towards conformational differences being the deciding factor in the selective $GABA_{B1a}$ and CHOP interaction. The coiled-coil domain of $GABA_{B1}$ was recently found to associate with the brain-specific RNA-binding protein Marlin-1. In addition, Marlin-1 binds to the 3`-untranslated regions of $GABA_{B1}$ and $GABA_{B2}$. Surprisingly, down-regulation of Marlin-1 resulted in an increase of $GABA_{B2}$ protein but did not change $GABA_{B1}$ protein levels (Couve et al., 2004). By regulating cellular levels of $GABA_{B2}$, Marlin-1 might thus control the expression levels of functional $GABA_B$ receptors. Msec7-1, a guanine-nucleotide-exchange factor (GEF) for the ADP-ribosylation factor (ARF) family of GTPases, has been implicated in many steps of vesicular membrane transport (Jackson and Casanova, 2000), and was shown to increase cell surface expression of $GABA_B$ receptors through interaction with the cytoplasmic LL motif of $GABA_{B1}$ (Restituito et al., 2005). Msec7-1 is involved in the export of proteins from the trans-Golgi network (TGN) and accordingly is proposed to regulate the transport of $GABA_B$ receptors from the TGN to the cell surface.

PDZ domain interactions

Many interactions with the $GABA_{B2}$ subunit involve a carboxyl-terminal motif (VSGL) that is able to interact with PDZ-domain containing scaffold proteins. PDZ (PSD-95/Discs-large/ZO 1) domains are 90-amino acid protein-protein interaction modules that recognize and bind to specialized motifs in the distal carboxyl termini of target proteins such as G protein-coupled receptors and ion channels. One of them, tamalin (for `tagging mGluRs and ARF-GEF`), a scaffolding protein that binds to the C-termini of type1 metabotropic glutamate receptors (mGluRs) was found in a yeast two-hybrid assay to interact with $GABA_{B2}$ (Kitano et al., 2002). The multi-PDZ domain protein Mupp1 binds with its PDZ13 domain to the $GABA_{B2}$ subunit and was recently shown to co-localize with $GABA_{B2}$ in heterologous and neuronal cells. Disruption of the binding decreased the stability of the receptor, which was followed by changes in receptor signaling (Balasubramanian et al., 2007). Suggestions for the mechanisms by which PDZ proteins decrease receptor stability may be a slowing down of the targeting rate to lysosomal and/or proteasomal compartments or blocking of proteolytic degradation of PDZ-binding partners. Interestingly, Mupp1 has previously been reported to interact with the serotonin 5-HT_{2C} receptor via its 10^{th} PDZ domain. As these three show overlapping distribution in the brain, Mupp1 might link $GABA_B$ and 5-HT_{2C} receptors together and thus facilitate cross-talk between $GABA_B$ and serotonin receptors (Slattery et al., 2005; Torres-Escalante et al., 2004).

Interaction with other receptors

Two prominent examples of the interaction of $GABA_B$ receptors with other neuronal receptors are $GABA_A$ and mGluR1 receptors. The γ2 subunit of $GABA_A$ receptors promotes surface expression of the $GABA_{B1}$ subunit, also in absence of $GABA_{B2}$ (Balasubramanian et al., 2004). $GABA_A\gamma2$ and $GABA_{B1}$ were shown to interact as single subunits, and also the γ2 subunit of the functional $GABA_A$ receptor was shown to bind to the $GABA_{B1,2}$ heterodimer. In this case, the γ2 subunit enhances $GABA_B$ receptor internalization upon agonist stimulation. This relationship would thus provide an activation-dependent feedback mechanism.

The crosstalk between $GABA_B$ and mGluR1 receptors has been implicated in the modulation of long-term depression (LTD) in the cerebellum. LTD of excitatory transmission at cerebellar parallel fiber-Purkinje cell synapses is a form of synaptic plasticity crucial for cerebellar motor learning. $GABA_B$ receptors in the dendritic spines of Purkinje cells are activated by GABA spillover from vicinal GABAergic interneurons. In cerebellar Purkinje cells $GABA_B$ receptors strongly co-localize with mGluR1 receptors and were shown to increase mGluR1 signaling (Kamikubo et al., 2007; Tabata et al., 2004).

RGS proteins

Two members of the Regulators of G-protein signaling (RGS) protein family, RGS2 and RGS12, have been described to modulate $GABA_B$ receptor functions. In contrast to other interacting molecules there is no evidence that RGS proteins directly bind $GABA_B$ receptors. RGS proteins are well known for their functions as GTPase activating proteins (GAP) and act through modulation of the GTPase activity of $G_{\alpha i/o}$ subunits. RGS proteins were later also found to regulate G-protein-effector interactions, though the mechanisms are not yet clear. For example, expression of RGS2 and its specific interaction with Kir3 account for the larger K^+ currents and stronger desensitization observed in dopaminergic when compared to GABAergic neurons (Labouebe et al., 2007). In the case of the multidomain protein RGS12, which contains a phosphpotyrosine binding (PTB) domain additional to its GAP domain, Diverse-Pierlussi and her group demonstrated that RGS12 binds via this PTB domain to the SNARE-binding region in loop II-III of the N-type calcium channel alpha1 subunit, that is phosphorylated upon $GABA_B$ receptor activation. In addition, binding of RGS12 was shown to accelerate $GABA_B$ mediated inhibition of the calcium channel, most likely through inhibition of the syntaxin-channel interaction which is important for the stabilization of the binding of the $G_{\beta\gamma}$ subunits to the calcium channels (Richman et al., 2005).

In summary, most screens for $GABA_B$ interacting molecules have so far been accomplished with yeast two-hybrid systems and have in majority revealed proteins that influence the biosynthesis, trafficking or surface expression of the receptor. But none of them could so far be shown to directly modulate receptor activity. Yeast two-hybrid screens might not be suitable to identify activity-modifying binding partners, as this technique carries several drawbacks. One constraint is that only parts of the receptor are used as baits, and so far only a limited number of receptor domains have been used to search for specific interacting proteins. The main reason for this may be that the functional receptor exists as a compact dimer of the $GABA_{B1}$ and the $GABA_{B2}$ subunit, which likely results in different conformations and the consequently exposure of different molecular interfaces for possible protein interactions in the assembled receptor compared to the individual subunits. Therefore, the identification of components of a macromolecular complex, as have been described e.g. for the Ca^{2+} channels (Dai et al., 2009), might require new proteomic approaches using native $GABA_B$ receptor heterodimers.

Therefore our group decided for a proteomic approach using affinity purification combined with high-resolution nanoflow liquid-chromatography tandem mass spectrometry to identify interaction partners of the $GABA_B$ receptor. Native $GABA_B$ receptors were purified from

membrane preparations prepared from total rat and mouse brains using antibodies against $GABA_{B1}$. Among the purified proteins were the $GABA_{B1}$ and $GABA_{B2}$ subunits, and proteins which are known to be involved in $GABA_B$ mediated signaling pathways, such as kinases like PKA, PKC and CaMKII, certain G-protein subunits and some known effector channels, like the voltage-gated Ca^{2+} channels. Purification of native $GABA_B$ receptor complexes revealed members of the K^+ channel tetramerization domain containing (KCTD) protein family, namely KCTD8, 12, 12b, and 16, associated with the $GABA_B$ receptor. However, none of the hitherto described $GABA_B$ interacting proteins were co-purified. This could be partially due to the fact, that we aimed to identify interactions with functional membrane-bound receptors and purified exclusively membrane fractions. However, the previously described, and mostly in Y2H screens discovered, interactions might occur predominantly in cytosolic or nuclear compartments, which imply that some of them could be false positive hits.

KCTD protein family

T1 domain as a structural determinant

The $GABA_B$ interacting proteins described in this dissertation constitute one subfamily of the potassium (K^+) channel tetramerization domain containing (KCTD) protein family. The name refers to the conserved domain at the N-terminus of these proteins, which is highly homologous to the tetramerization (T1) domain of some voltage-gated K^+ (Kv) channels. Four Kv subunits assemble to form a functional channel. The N-terminal tetramerization domain contains a BTB (Bric-a-brac, Tramtrack, Broad-complex) domain, also designated POZ (Pox virus and Zinc finger) domain, that fold and mediates subunit specific assembly. The BTB domain is a widely distributed protein-protein interaction module, which derives its name from the observation by Laski and colleagues that the Drosophila transcription factors Bric-a-brac, Tramtrack, and Broad Complex display a region of sequence similarity at their N-terminus that they named the BTB domain (Godt et al., 1993; Zollman et al., 1994). At the same time, Bardwell and Treisman realized that some Poxvirus proteins have resemblance to a portion of the Zinc finger proteins; they named this region the POZ (Pox virus and Zinc finger) domain (Bardwell and Treisman, 1994). These identical motifs define the BTB/POZ domain, but are collectively abbreviated as a BTB domain. BTB domains can mediate both self-association as well as interaction with non-BTB partners. Proteins that contain a BTB domain participate in many biological functions including transcriptional regulation (Melnick et al., 2000), protein degradation (Furukawa et al., 2003), cytoskeletal regulation and ion channel function (Stogios et al., 2005). Still, a common feature that emerges from all BTB

domains is that they allow multiple copies of a protein to associate with each other, thus amplifying or modulating their functions.

KCTD family members – functions and implications in disease

The family of KCTD genes contains 21 and 22 members in the human and mouse genome, respectively. The regions encoding the N-terminus of all family members are highly homologous. The C-terminal regions show large differences in length and as well in amino acid sequences. According to their homology, KCTD proteins can be grouped into subfamilies as indicated in the phylogenetic tree in Figure 1-2. Since our work began on the characterization of KCTD8, 12, 12b, and 16 in respects to $GABA_B$ receptor interaction and function in 2004, a considerable number of studies on other KCTD family members has emerged. Often conserved protein families with large constituents are divided into subfamilies that share similar functions, but are compartmentalized temporally and/or spatially. Knowledge about the different members of the KCTD protein family, their structural and functional properties might elucidate the full spectrum of actions inherited by the $GABA_B$ interacting KCTD subfamily.

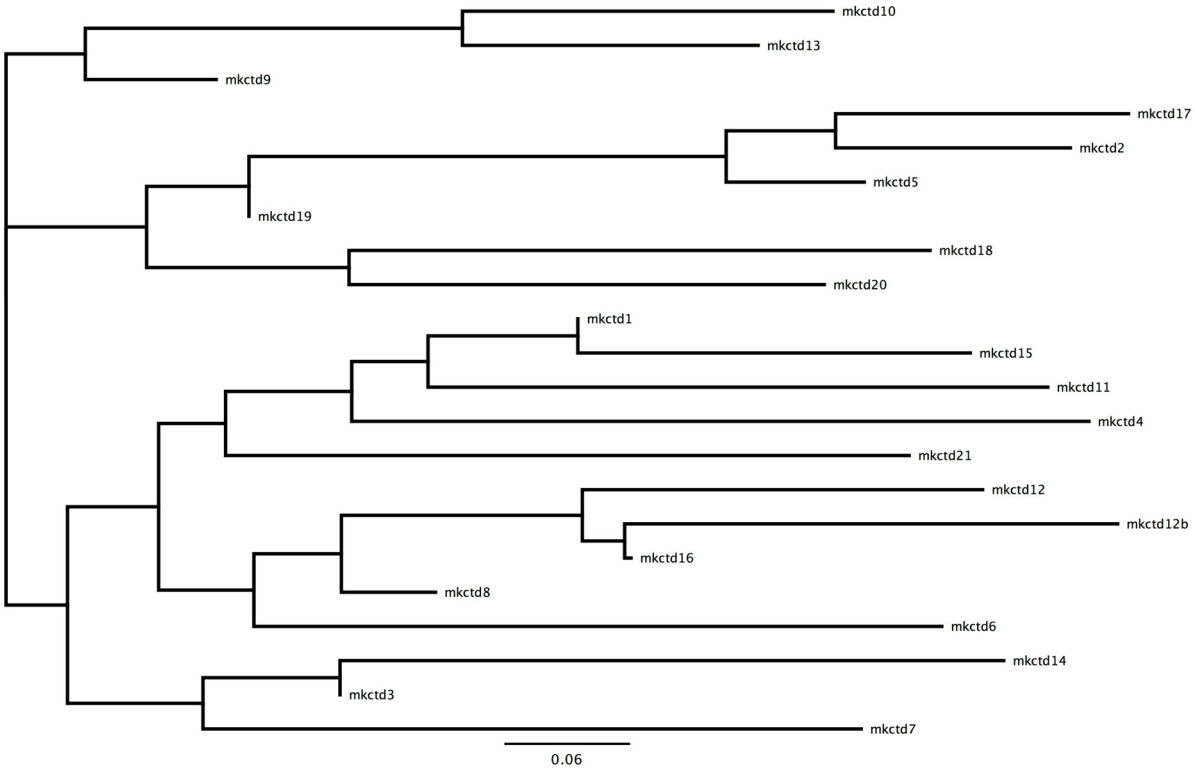

Figure 1-2: Phylogenetic tree showing the sequence relationships between members of the KCTD protein family. Mouse sequences were aligned by using Clustal method. The phylogenetic tree was derived from the sequence comparisons using the neighbor-joining method in Clustal X. The scale bar represents 0.06 amino acid substitution per site.

KCTD 10 and 13 (Polymerase delta-interacting protein 1(PDIP1)), together with Tumor necrosis factor, alpha-induced protein 1 (TNFAIP1), constitute one subgroup of KCTDs. Apart from the T1 domain, these proteins share a proliferating cell nuclear antigen (PCNA) binding motif (QTKV-EFP) at the C-terminus. They interact with PCNA and the small subunit (p50) of DNA polymerase δ, and stimulate polymerase δ activity in the presence of PCNA. All three were reported to be TNF-α inducible and therefore suggested to participate in TNF-α induced DNA replication/repair pathways (Wang et al., 2009; Zhou et al., 2005a; Zhou et al., 2005b).

KCTD 2, 5, and 17 constitute another subgroup with KCTD5 being the sole member characterized. KCTD 5 was identified by Weger et al. as an interaction partner of the large regulatory proteins Rep78/Rep68 of adeno-associated virus type 2. In addition, they found wildtype Rep78/Rep68 proteins inducing the translocation of KCTD5 into the nucleus (Weger et al., 2007). In 2008 Bayon et al. showed that KCTD5 interacts specifically with cullin3 ubiquitine ligase and might act as a substrate-specific adaptor that recruits proteins for ubiquitiniation and its subsequent degradation. Moreover, they showed that the BTB domain of KCTD5 mediates protein oligomerization (Bayon et al., 2008). KCTD5 is the first member

of the KCTD family, which was characterized by x-ray chrystallography. Unexpectedly the structure revealed a pentameric assembly and leads to speculations that the BTB domain in KCTD5, and most likely KCTD2 and 17, forms a pentamer. This is in direct contrast to the T1 domains of Kv channels which form a tetramer. A difference of four amino acids seems to be responsible for the different structures observed (Dementieva et al., 2009).

KCTD1 is a nuclear protein that mediates transcriptional repression and homomeric interactions and specifically acts as a negative regulator of the transcription factor AP-2α (Ding et al., 2008). In addition, Ding et al. also found that KCTD1 interacts with three major members of the AP-2 family and inhibits their transcriptional activities (Ding et al., 2009). In the same study, the authors further showed that homomeric and heteromeric interaction is mediated via the BTB domain.

KCTD 11 was one of the first members of the KCTD family to be described. The group of Alberto Gulino identified KCTD11 as a suppressor of sonic hedgehog homolog (Shh) signaling and investigated its role in the formation of medulloblastomas. Chromosome 17p deletion, the most frequently detected genetic lesion in medulloblastoma, leads to the loss of KCTD11, which in turn might lead to a deregulation of the tumor-promoting Shh pathway in medulloblastoma. In this context Argenti et al. found that KCTD11 can antagonize the Hedgehog pathway which in turn regulates proliferation and apoptosis of developing granule cell progenitors. And the BTB domain seems to be necessary for this effect (Argenti et al., 2005).

In a recent study, changes in the KCTD3 gene were suggested to be one of the causes in patients with autism spectrum disorder (Poot et al., 2009). Moreover KCTD3 was recognized as an antigen in patients with renal-cell carcinoma (therein called renal carcinoma antigen NY-REN-45), but further evidence for an emerging role in the pathology is lacking (Scanlan et al., 1999). KCTD3, however, is unique in that it contains, apart from the BTB domain, several nuclear targeting signals and a single transmembrane domain (Scanlan et al., 1999).

Recently, several large scale genetic screens have identified different KCTDs as markers for different pathologies. Expression of KCTD 9 was found to be upregulated in patients with severe hepatitis B (Zhou et al., 2008). KCTD 15 was identified in a screen for gene loci with single-nucleotide polymorphisms that are associated with obesity (Cauchi et al., 2008; Zhao et al., 2009). Van Bogaert et al. reported a mutation of KCTD7 in patients suffering from progressive myoclonic epilepsy caused by neurodegeneration (Van Bogaert et al., 2007). Despite the genetic links, however, underlying biological functions and mechanisms remain unknown. KCTD20 was recently identified in Hispanic-American participants from the Insulin Resistance Atherosclerosis Family Study as a candidate gene that may be associated in the

acute insulin response to glucose, which is used as a predictor of type 2 diabetes (Rich et al., 2009). Also KCTD12 was found in a screen for single nucleotide polymorphisms associated with type 2 diabetes (Cauchi et al., 2008). However, the true contribution of these KCTDs on incidences of type 2 diabetes still needs to be verified.

KCTD12 was first cloned from a fetal cochlea library and described by Resendes et al. to be expressed in multiple cells in murine and human cochlea and suggested to play a role in hearing loss (Resendes et al., 2004). Furthermore, Kawai et al. performed an extensive protein expression study of bone and soft tissue sarcomas in order to develop novel diagnostic and therapeutic biomarkers and allow molecular classification of the tumors. In their study, KCTD12 was identified as one of the novel tumor markers. Notably, herein KCTD12 was identified as a promising prognostic marker for the separation of gastrointestinal stromal tumor patients into low and high risk groups. Identification of low-risk patients adds important value to their therapy as they could profit either from adjuvant imatinib treatment or from the possibility of minimizing the risk of unnecessary treatment (Kawai et al., 2008).

Due to their sequence homology KCTD12 is phylogenetically grouped with KCTD12b, KCTD8, and KCTD16. Three of the six zebrafish orthologs of this KCTD subfamily Lov, Ron, and Dex are expressed asymmetrically in zebrafish habenular neurons and their projections to the interpeduncular nucleus. Although these left-right differences are not observed in higher vertebrates, the zebrafish studies might lead to the understanding of the development and evolution of lateralized features of the vertebrate nervous system (Gamse et al., 2005). Moreover these studies suggest a developmental role for KCTD 12. The identification and characterization of KCTD8, 12, 12b, and 16 as novel interaction partners of the $GABA_B$ receptor is described in detail in this thesis.

Aim of the thesis

Pharmacological and functional differences of $GABA_B$ receptors observed in vivo suggest a wider molecular repertoire than the one provided by the two currently known receptor subtypes $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$. Alternative explanations, therefore, may be the existence of yet unidentified interacting proteins capable of modifying receptor activity, pharmacology, and localization. This work was initiated when a proteomics' approach using native membrane bound $GABA_B$ receptors from mouse brain in an affinity purification experiment had isolated putative binding partners of the receptor. We decided to focus on four sequence-related proteins – KCTD8, 12, 12b, and 16 - that were co-purified with the receptor.

This dissertation attempts to characterize these novel $GABA_B$ receptor interacting proteins including the analysis of structural distinctions between these proteins as well as their functional significance, the identification of the critical binding sites on the receptor and each KCTD protein, the investigation of the functional consequences with respect to $GABA_B$ receptor action, and the examination of the spatial and temporal distribution pattern of the $GABA_B$ receptor interacting KCTDs.

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2 Native $GABA_B$ receptors are heteromultimers with a family of auxiliary subunits

(submitted)

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Abstract

The γ-aminobutyric acid receptor B ($GABA_B$) is a heteromeric G-protein coupled receptor that is thought to assemble and signal through the two subunits $GABA_{B1}$ and $GABA_{B2}$. Here we show by functional proteomics that $GABA_B$ receptors in the rat brain are high molecular weight complexes of $GABA_{B1}$, $GABA_{B2}$ and members of a subfamily of the 'potassium channel tetramerization domain-containing' (KCTD) proteins. KCTD proteins 8, 12, 12b and 16 exhibit distinct expression profiles in the brain and tightly associate with the C-terminus of $GABA_{B2}$ as tetramers. This coassembly changes the properties of the $GABA_{B(1,2)}$ core receptor: the KCTD proteins increase agonist potency and markedly alter the G-protein signaling of the receptors by accelerating onset and promoting desensitization in a KCTD subtype-specific manner. Together, our results establish the KCTD proteins as auxiliary subunits of $GABA_B$ receptors that determine the pharmacology and kinetics of the receptor response.

Introduction

$GABA_B$ receptors are the G-protein-coupled receptors (GPCRs) for γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian CNS (Bettler et al., 2004). $GABA_B$ receptors are expressed in virtually all neurons in the brain (Kaupmann et al., 1998) and influence synaptic transmission and signal propagation by regulating the activity of voltage-activated Ca^{2+} (Cav) and inward-rectifier K^+ (Kir) channels via the βγ dimer of the activated G-protein. Inhibition of Cav2 channels suppresses neurotransmitter release and blocks dendritic Ca^{2+} spikes, while activation of Kir3 channels induces slow inhibitory postsynaptic potentials and mediates shunting inhibition (Perez-Garci et al., 2006, Luscher et al., 1997). The time course and/or pharmacology of $GABA_B$-mediated current responses vary among neurons (Cruz et al., 2004) and between pre- and postsynaptic effectors (Deisz et al., 1997), suggesting the existence of a variety of molecularly distinct receptor subtypes (Bonanno and Raiteri, 1993). However, molecular cloning revealed only two types of receptors; these are generated by isoforms of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann et al., 1997), which must coassemble with $GABA_{B2}$ to form functional $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors (Kaupmann et al., 1998). Both types of receptors exhibit identical pharmacological and kinetic properties (Ulrich and Bettler, 2007) and differ approximately ten-fold in agonist-affinity from their native counterparts (Kaupmann et al., 1998).

At present, the differences in kinetics and pharmacology observed between responses of native $GABA_B$ receptors are unresolved as are the mechanism(s) introducing functional

diversity into heterologously expressed $GABA_{B(1,2)}$ receptors. Protein phosphorylation (Couve et al., 2002), 'regulator of G-protein signaling' (RGS) proteins (Labouebe et al., 2007) or distinct composition of effector channels (Cruz et al., 2004) have been proposed to underlie at least some of these differences. Alternatively, native $GABA_B$ receptors may contain additional, as yet unknown subunits that modify their properties. To test this hypothesis a recently developed proteomic approach combining affinity purification of appropriately solubilized protein complexes and quantitative mass spectrometric analysis of their constituents (Berkefeld et al., 2006; Schwenk et al., 2009; Zolles et al., 2009) was applied to $GABA_B$ receptors.

From this analysis, four sequence-related members of the 'potassium channel tetramerization domain-containing' (KCTD) family of proteins were identified as intrinsic auxiliary subunits of native $GABA_B$ receptors. These KCTD proteins exhibit distinct expression patterns in the brain and endow $GABA_B$ receptors with subtype-specific G-protein signaling regarding activation and desensitization kinetics as well as agonist potency. The KCTD-mediated expansion of the functional repertoire provides a molecular basis for the functional diversity of heterologously expressed and native $GABA_B$ receptors.

Results

Proteomic analysis of $GABA_B$ receptors from mouse brain

For proteomic analysis of native $GABA_B$ receptors, we performed affinity-purifications (APs) using antibodies specific for either of the two core subunits $GABA_{B1}$ (anti-$GABA_{B1}$ targeting $GABA_{B1a}$ and $GABA_{B1b}$) or $GABA_{B2}$ (anti-$GABA_{B2}$) on appropriately solubilized membrane fractions prepared from total rat and mouse brains. The $GABA_B$ receptors in these protein preparations exhibited high molecular weights of more than ~0.75 MDa as visualized by two-dimensional gel separations using blue native polyacrylamide gel electrophoresis (BN-PAGE) and subsequent denaturing SDS-PAGE (Figure 2-1a). Total eluates of anti-$GABA_B$ APs from brain membrane fractions of rats and wildtype (WT) mice as well as of mice with targeted deletions of the receptor core subunits (Gassmann et al., 2004; Schuler et al., 2001; Vigot et al., 2006) were analyzed by high-resolution nanoflow liquid-chromatography tandem mass spectrometry (nano-LC MS/MS). These analyses provided the identity of the proteins in the respective eluates as well as quantitative data on their amounts as determined from the extracted ion current peak-volumes (Figure 2-1b) of multiple tryptic peptides (Cox and Mann, 2008; Schwenk et al., 2009). The results of these MS-analyses showed that both $GABA_{B1}$ and $GABA_{B2}$ were purified by either anti-$GABA_B$ antibody at high yield (rPV score (see Methods), Figure 2-2b) with the large number of peptides retrieved by mass

spectrometry covering 69% and 61% of the accessible primary sequence of $GABA_{B1}$ and $GABA_{B2}$, respectively (S Figure 2-1a). Moreover, normalization of the peak-volumes to the number of MS-accessible amino acids ($abundance_{norm}$) showed that the two core subunits were present in both anti-$GABA_B$ APs at equimolar ratios (Figure 2-2a,b).

In addition to $GABA_{B1}$ and $GABA_{B2}$, our MS-analyses identified the KCTD proteins 8, 12, 12b, and 16, four closely related members of a large family of soluble proteins (21 and 22 different genes in the human and mouse genome, respectively) that share conserved domains in their N- (T1-domain, homology to Kv-type K^+ channels) and C-termini (C-terminal domain, Figure 2-1c, S Figure 2-2). KCTD isoforms 8, 12, and 16 were copurified with both anti-$GABA_B$ antibodies from rats and WT mice at similarly high yields as $GABA_{B1}$ and $GABA_{B2}$ ($abundance_{norm}$ values, Figure 2-2a, b; sequence coverages of 79%, 97% and 80%, respectively; for details see S Figure 2-1b), while KCTD12b was retrieved in markedly smaller amounts (Figure 2-2b). No KCTD-specific peptides were detected in APs with anti-$GABA_{B1}$ (Figure 2-1b) and anti-$GABA_{B2}$ (not shown) from brains of $GABA_{B1}$-/- and $GABA_{B2}$-/- knock-out animals, respectively. In addition, KCTD proteins were not retrieved in anti-$GABA_{B1}$ APs from $GABA_{B2}$-/- brains, where MS-analyses detected considerable amounts of $GABA_{B1}$ protein (Figure 2-1b). Together, these results strongly suggested that KCTD proteins 8, 12, 12b, and 16 are integral constituents of native $GABA_B$ receptors via interactions with the $GABA_{B2}$ subunit.

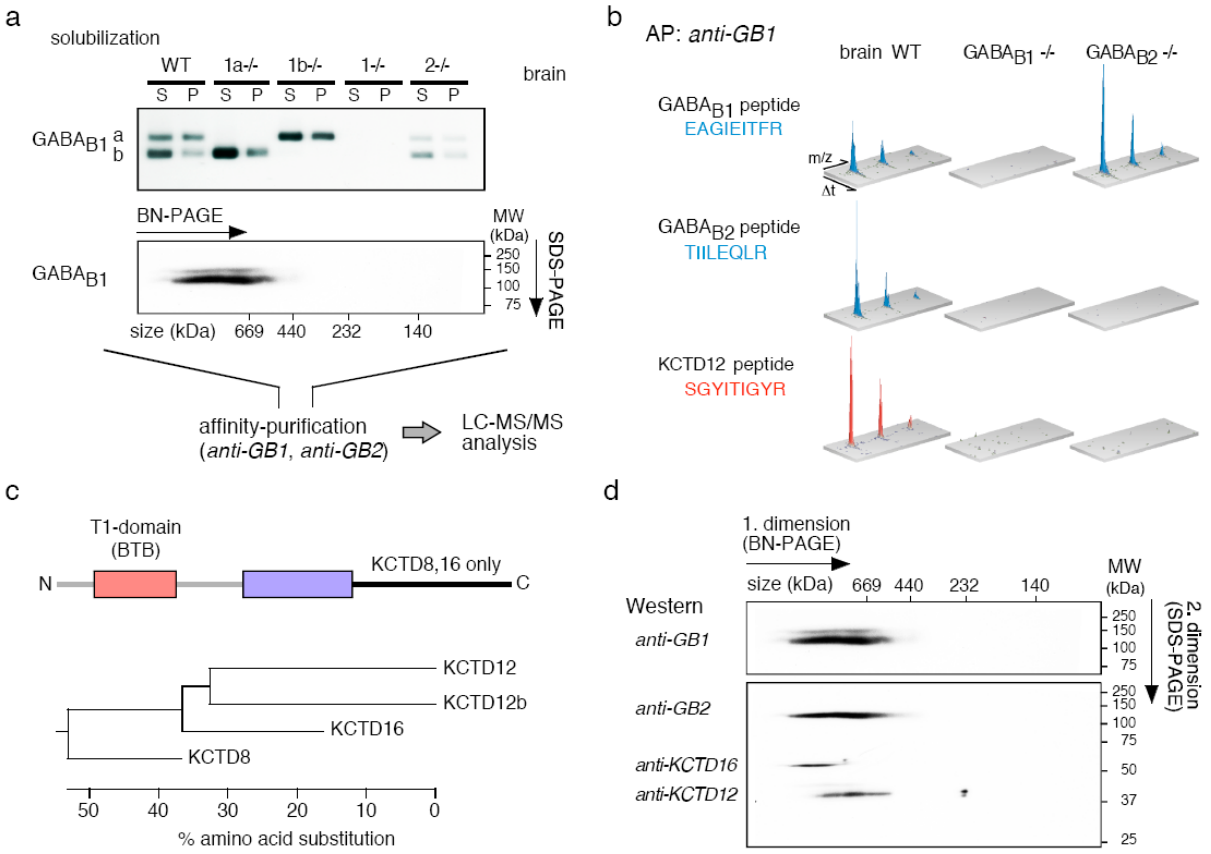
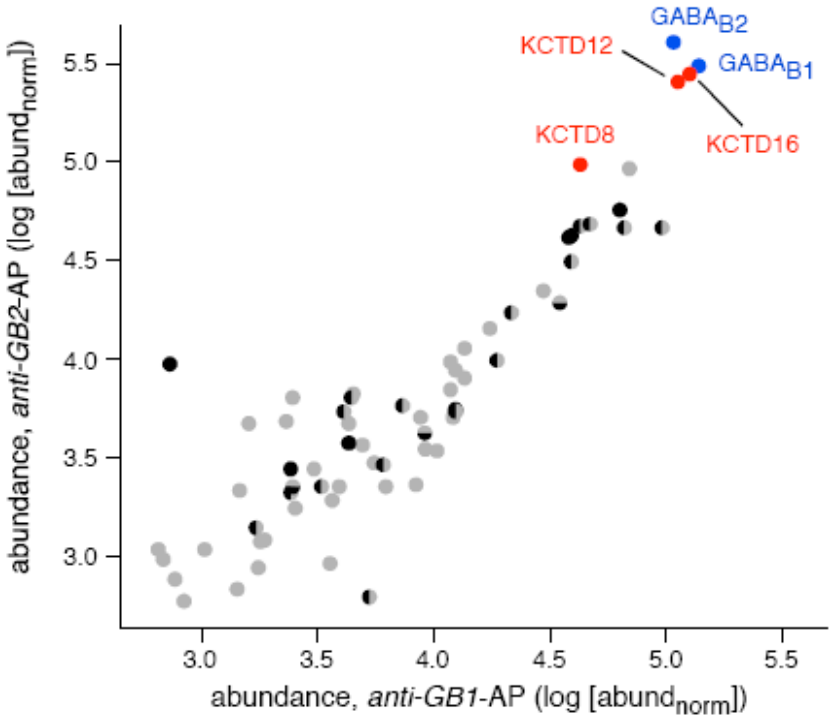


Figure 2-1: Proteomic analysis identifies four KCTD proteins as subunits of native $GABA_B$ receptors.

a, Scheme of the proteomic approach used for analysis of $GABA_B$ receptors from total rat and mouse brain. Upper panel: SDS-PAGE separation of solubilized (S) and insoluble (P) protein fractions obtained with the solubilization buffer CL-91 from brain membrane fractions of wildtype (WT) and the indicated knock-out mice. Lower panel: Two-dimensional gel separation of solubilized $GABA_B$ receptors from rat brain. Size (BN-PAGE) and molecular mass (SDS-PAGE) as indicated. Western blots in both panels were probed with the anti-$GABA_{B1}$ antibody. Solubilized membrane fractions were used for APs with the indicated antibodies, total eluates of these APs were analysed by nano LC-MS/MS. **b**, Peak-volumes (integral of MS signal intensity in the m/z-retention time plane; normal and isotope peaks) of the indicated peptides (z = +2, no modifications) determined from MS-spectra during nano LC-MS/MS analysis of anti-$GABA_{B1}$ APs from brain membrane fractions of WT and the indicated knock-out mice. Note that the KCTD12 peptide was only obtained from WT material. **c**, Structural scheme illustrating the conserved domains and dendrogram (Clustal method) of the identified subfamily of KCTD proteins. **d**, Two-dimensional gel separation as in (a) Western blotted with the indicated antibodies. Note the close co-segregation of KCTD proteins 12 and 16 with $GABA_{B1}$ and $GABA_{B2}$.

a



b

	anti-GB1		*anti-GB2*		*anti-KCTD12*		*anti-KCTD16*	
Protein ID	rPV	log (abund$_{norm}$)	rPV	log (abund$_{norm}$)	rPV	log (abund$_{norm}$)	rPV	log (abund$_{norm}$)
GABA$_{B1}$	785	5.14	2048	5.49	1628	5.34	2723	5.73
GABA$_{B2}$	1762	5.03	2490	5.61	3226	5.58	7377	5.86
KCTD8	112	4.63	288	4.99	1619	5.97	5366	5.91
KCTD12	429	5.10	1018	5.45	34262	6.75	6472	6.49
KCTD12b	n.d.	n.d.	20	3.15*	1001	5.14*	n.d.	3.36*
KCTD16	219	5.05	884	5.41	2668	6.09	15419	6.45

Figure 2-2: GABA$_B$ receptors are robustly associated with a subset of KCTD proteins in the rodent brain.

a, Double logarithmic plot of normalized abundance (abundance$_{norm}$) values determined for proteins in APs with anti-GABA$_{B1}$ and anti-GABA$_{B2}$. Grey circles denote unspecific background proteins, filled circles are proteins specifically copurified with both antibodies, while half-filled symbols represent proteins specifically copurified with only one of them (left side filled with anti-GABA$_{B2}$, lower half filled with anti-GABA$_{B1}$). **b**, Relative peak volumes (rPV, see Methods) and logarithmic abundancenorm values of the GABA$_B$ receptor core subunits and the KCTD proteins 8, 12, 12b, and 16 obtained in APs from rat brain membranes with the indicated antibodies. rPV values > 10 indicate specific copurification; values with asterisks refer to abundance$_{norm}$ values corrected for homology between KCTD isoforms 12 and 12b.

Coassembly of KCTD proteins with native and heterologously expressed $GABA_B$ receptors

Coassembly of the identified KCTD proteins with native $GABA_B$ receptors was further corroborated by reverse APs using antibodies specific for either KCTD12 (*anti-KCTD12*; S Figure 2-3) or KCTD16 (*anti-KCTD16*; S Figure 2-3), the most abundant KCTDs in the anti-$GABA_B$ APs (Figure 2-2), on membrane fractions from rat and mouse brain. MS-analyses of the respective eluates showed that both anti-KCTD antibodies, besides enriching their target proteins, effectively copurified $GABA_{B1}$ and $GABA_{B2}$ as wells as the other KCTD isoforms (Figure 2-2b). In fact, the two $GABA_B$ receptor core subunits were the most abundantly copurified proteins apart from KCTDs and the only GPCR subunits detected by mass spectrometry. The KCTD-$GABA_B$ coassembly was further confirmed by Western blot analysis of two-dimensional gel separations of brain membrane fractions using the anti-$GABA_B$ and anti-KCTD antibodies. As illustrated in Figure 2-1d, both KCTD12 and KCTD16 strictly co-migrated with the $GABA_B$ core subunits, although their signals exhibited only partial overlap with each other. This suggests that a portion of both KCTDs may be integrated into the same $GABA_B$ complexes, while the remaining KCTD proteins 12 and 16 participate in distinct $GABA_B$ receptors of either higher (KCTD16) or lower (KCTD12) molecular size (Figure 2-1d). Interestingly, all $GABA_B$ receptors resolved on native gels appeared associated with the KCTD proteins and markedly exceeded the molecular weight predicted for $GABA_{B(1,2)}$ heterodimers (~250 kDa). These results together with the abundant copurification pointed towards direct coassembly of the KCTD proteins with $GABA_B$ receptors. This was tested in APs from membrane fractions prepared from cultured HEK293 cells that heterologously expressed various combinations of differentially tagged $GABA_{B1}$ (Myc-tag), $GABA_{B2}$ (HA-tag) and KCTD12 (FLAG-tag) proteins. The results showed that the KCTD-$GABA_B$ coassembly is mediated by direct interaction between the KCTD and $GABA_{B2}$ proteins (Figure 2-3a), in line with the AP results from knock-out animals (Figure 2-1b).

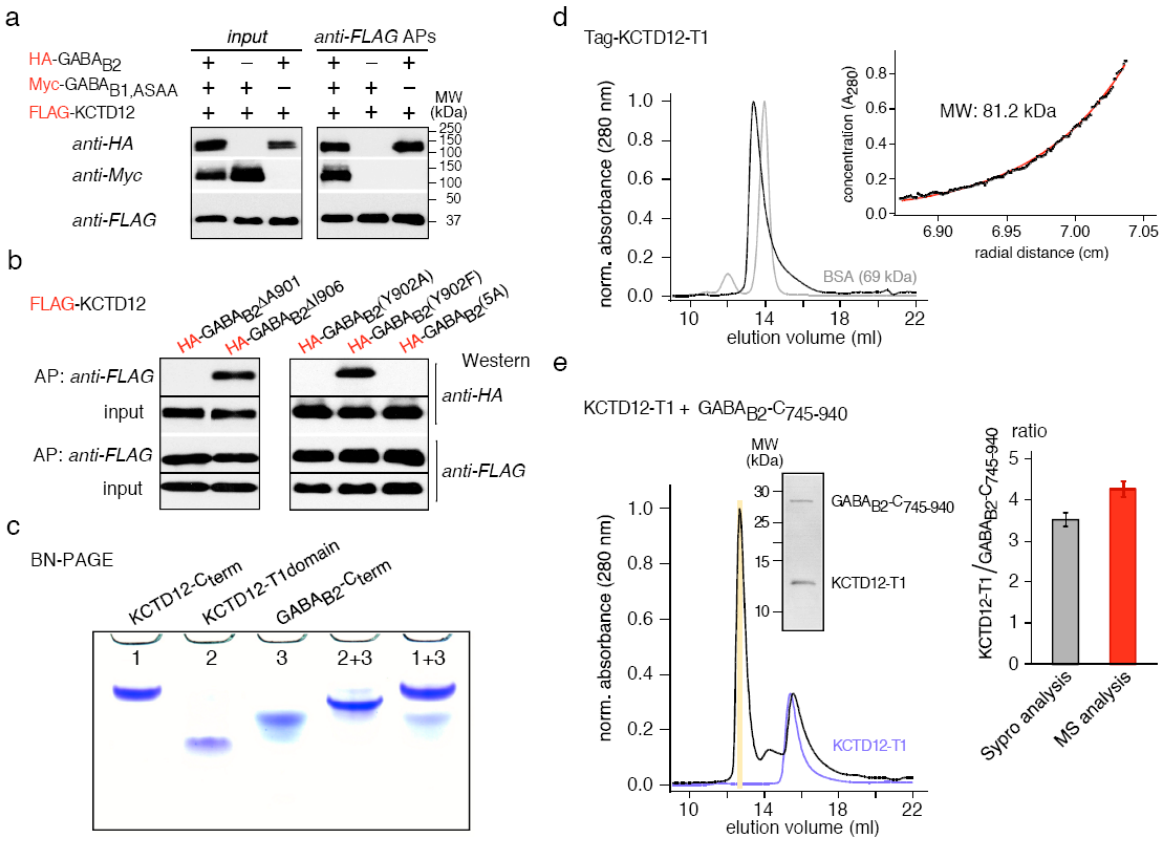


Figure 2-3: Molecular interface and stoichiometry of the $GABA_B$-KCTD interaction.
a, SDS-PAGE separation of input and eluate of anti-FLAG APs Western blotted with the indicated antibodies. Input are membrane fractions from HEK293 cells coexpressing combinations of differentially tagged $GABA_{B1}$ (Myc-tag), $GABA_{B2}$ (HA-tag) and KCTD12 (FLAG-tag). The $GABA_{B1}$(ASAA) mutant was used to prevent retention of the protein in the endoplasmic reticulum (Pagano et al., 2001). Note selective interaction between KCTD12 and the $GABA_{B2}$ protein. **b**, Experiment as in (a) using FLAG-tagged KCTD12 and HA-tagged C-terminal deletions or point mutations of $GABA_{B2}$ as input. Similar results were obtained with KCTDs 8, 12b, and 16 (data not shown). **c**, Native PAGE separation of a binding assay using bacterially synthesized and purified T1- and C-terminal domains of KCTD12 (KCTD12-T1, KCTD12-C_{term}), and the C-terminus of $GABA_{B2}$ (aa 849-940). Proteins were visualized by Coomassie staining. Note that binding only occurred between KCTD12-T1 and $GABA_{B2}$-C_{term}. **d**, Size exclusion chromatography (SEC) of the GB1-tagged KCTD12-T1-domain (Tag-KCTD12-T1); pure BSA (MW of 69 kDa) was added for comparison and size calibration. Inset: Sedimentation equilibrium analysis of the monodisperse SEC fraction of Tag-KCTD12-T1 (protein MW of 20.4 kDa); data were obtained with a protein concentration of 0.5 mg/ml at 12.000 rpm. Continuous line represents fit of the data yielding the indicated MW. **e**, SEC analysis of the bacterially synthesized and purified complex of KCTD12-T1 and the $GABA_{B2}$ C-terminus (aa 745-940); purified KCTD12-T1 was added for comparison. Left inset: SDS-PAGE of the indicated SEC-fraction (KCTD12-T1+$GABA_{B2}$-$C_{745-940}$, yellow bar); the gel was stained with Sypro Orange. Right inset: Stoichiometry of the complex constituents obtained either from fluorescence intensity measurements (Sypro analysis) or from quantitative MS-analyses of gel separations as in the left inset; data are mean ± SD of 3 experiments. Note that both methods yielded a stoichiometry of about 4:1 for KCTD12-T1 : $GABA_{B2}$-$C_{745-940}$.

Structural determinants and stoichiometry of the $GABA_B$-KCTD interaction

The structural determinants that mediate the interaction between KCTD proteins and $GABA_{B2}$ were identified by APs from HEK293 cells coexpressing FLAG-tagged KCTD12 and a series of deletion and point mutants of HA-tagged $GABA_{B2}$. These experiments revealed a six amino acid stretch (amino acids 901-906) at the very C-terminus of the $GABA_{B2}$ protein as a critical interaction determinant, with an aromatic residue at position 902 being of particular importance (Figure 2-3b). Tyrosine (WT) or phenylalanine at this position promoted interaction with KCTD12, while replacement with alanine abolished it (Figure 2-3b). Subsequent binding assays with the C-terminus of $GABA_{B2}$ (amino acids 849-940) and the conserved structural domains of KCTD12 (Figure 2-1c), all synthesized in bacteria and purified to homogeneity, were used to identify the interaction site(s) on the KCTD proteins. Native gel separations of these samples showed that the T1-domain of KCTD12 (KCTD12-T1) robustly assembled with the $GABA_{B2}$ C-terminus, whereas the C-terminal domain of KCTD12 failed (Figure 2-3c).

To investigate the stoichiometry of the $GABA_B$ receptor components, purified GB1-tagged KCTD12-T1 (Tag-KCTD12-T1) as well as purified complexes of KCTD12-T1 and the full-length C-terminus of $GABA_{B2}$ (KCTD12-T1+$GABA_{B2}$-$C_{745-940}$), obtained by bacterial coexpression, were analysed by size-exclusion chromatography, analytical ultracentrifugation and quantitative mass spectrometry. As shown in Figure 2-3d, the tagged KCTD12-T1 eluted as a monodisperse protein fraction in size-exclusion chromatography and, in subsequent sedimentation equilibrium analyses, displayed a molecular weight of 82.9 ± 4.3 kDa (mean ± SD of 4 different runs, see Methods), a value very close to the weight calculated for a tetramer of Tag-KCTD12-T1 proteins (weight of the monomer is 20.4 kDa). Similarly, the KCTD12-T1+$GABA_{B2}$-$C_{745-940}$ complex, purified via the KCTD12-T1 subunit, eluted as a monodisperse peak in size-exclusion chromatography. Analysis of its subunit stoichiometry via mass spectrometry- or fluorescence-based quantification (see Methods) following separation by SDS-PAGE revealed a ratio for KCTD12-T1 : $GABA_{B2}$-$C_{745-940}$ of approximately 4:1 (Figure 2-3e). This result confirmed the tetrameric assembly of KCTD12-T1 and suggested that each KCTD-T1 tetramer offers one binding site for the C-terminus of $GABA_{B2}$.

Together, proteomic and biochemical analyses indicated that the KCTD proteins 8, 12, 12b and 16 are integral constituents of native $GABA_B$ receptors through intimate association of KCTD homo- and/or heterotetramers with the C-terminus of the $GABA_{B2}$ subunit. When related to the size on BN-PAGE gels, this arrangement suggests that native $GABA_B$

receptors are most likely dimeric assemblies (Maurel et al., 2008) of monomers made up of $GABA_{B1}$, $GABA_{B2}$ and a KCTD-tetramer (estimated monomer weight of ~350-400 kDa).

Expression pattern of KCTD proteins in the CNS

Next, the expression profiles of the four identified KCTD proteins in the CNS were investigated by in-situ hybridization. As illustrated in Figure 2-4a, the four KCTDs exhibited distinct, but partially overlapping expression patterns. Transcripts encoding the KCTD proteins 12 and 16 were detected throughout most regions of the brain, with particular abundance in the neocortex, the hippocampal formation (granule cells in the dentate gyrus, pyramidal cells in the CA1/CA3 regions), the cerebellum (Purkinje cells (KCTD12), Golgi cells (KCTD16)) and the septum (KCTD12). KCTD8 mRNA was predominantly observed in the olfactory bulb and in the granule cell layer of the cerebellum, whereas transcripts for KCTD12b appeared restricted to the medial habenula (Figure 2-4a). The subcellular distribution of the KCTD proteins 12 and 16 was assessed by the anti-KCTD antibodies used in pre-embedding immuno electron microscopy (EM) and immunolabeling of freeze-fracture replicas of the hippocampal CA1 region (stratum radiatum). Both techniques localized the *anti-KCTD12* and *anti-KCTD16* immunoreactivity to the plasma membrane of pre- and postsynaptic compartments of putative principal cells that were also stained with the anti-$GABA_{B1}$ antibody (Figure 2-4b, c). Thus, abundant *anti-KCTD12*/*16* immunoreactivity was found at the peri- and extrasynaptic membranes of dendritic shafts and spines of pyramidal cells and at the presynaptic membrane specialization of putative glutamatergic synapses (Figure 2-4b, c). Interestingly, in all locations immunoparticles revealed a similarly clustered organization for $GABA_{B1}$, KCTD12 and KCTD16 (Figure 2-4b, c), consistent with the known clustering of native $GABA_B$ receptors (Kulik et al., 2006) and their heteromultimeric assembly from these subunits reported here.

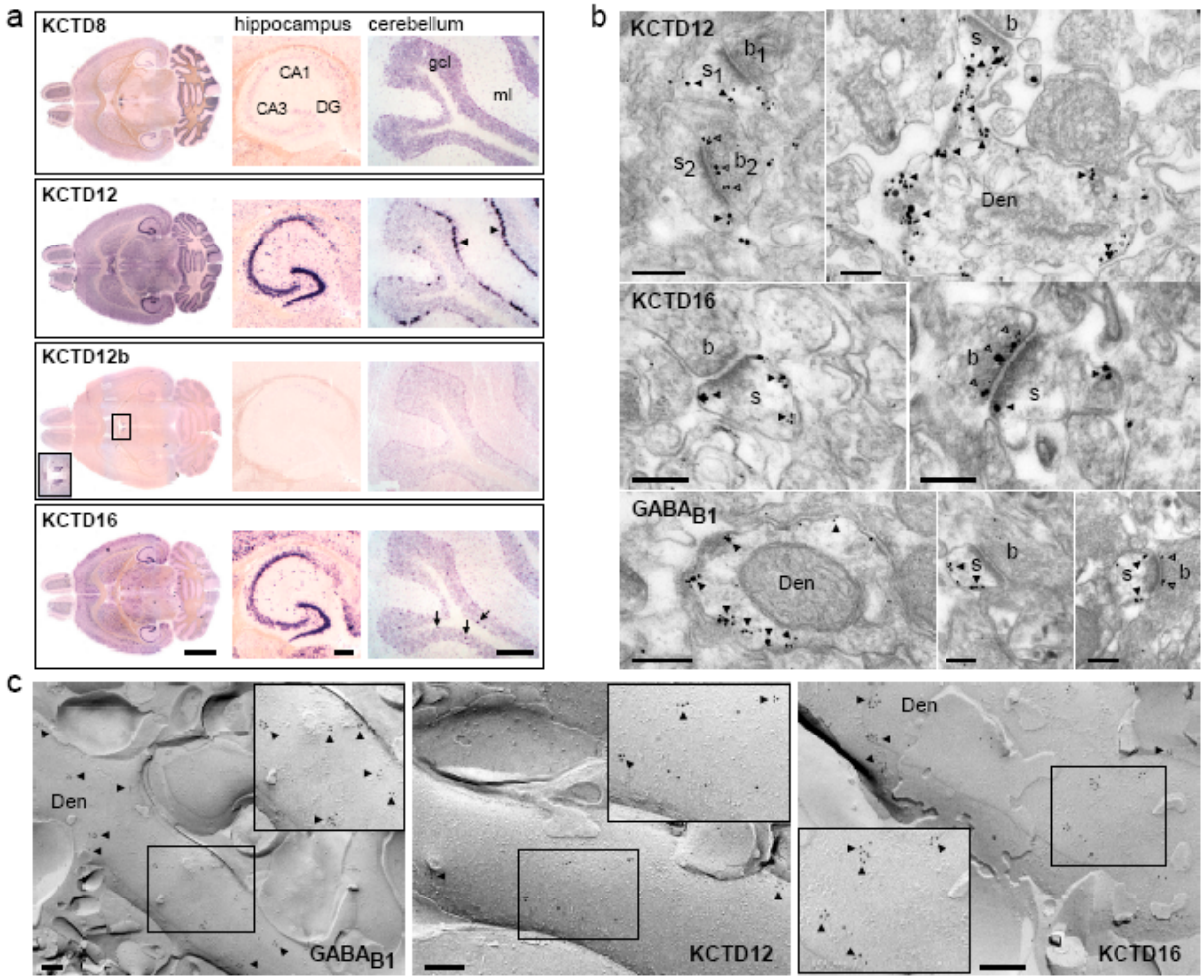


Figure 2-4: Expression profile and subcellular localization of KCTD proteins in the brain.

a, Distribution of transcripts encoding the indicated KCTD proteins in the adult mouse brain as obtained by *in-situ* hybridization with digoxigenin-labelled antisense probes on horizontal sections of the medial tier of the brain (overview (left), hippocampal formation (middle) and cerebellum (right)). Transcripts of KCTD proteins 12 and 16 are found throughout the brain with particular abundance in the neocortex and hippocampus; note the distinct pattern in the cerebellum (KCTD12 in Purkinje cells (arrowheads), KCTD16 in Golgi cells (arrows). KCTD8 mRNA is detected in the olfactory bulb and the granule cell layer of the cerebellum, whereas transcripts for KCTD12b appeared restricted to the medial habenula (open frame, enlarged in inset). DG, dentate gyrus; gcl, granule cell layer; ml, molecular cell layer. Scale bars are 200 μm (overviews) and 40 μm (hippocampus and cerebellum).

b, Electron micrographs of immunoreactivities for the indicated KCTD proteins and $GABA_{B1}$ in the stratum radiatum of the hippocampal CA1 region detected by pre-embedding immunogold EM. Immunogold particles were most abundant at the extrasynaptic plasma membrane (filled arrowheads) of dendritic spines (s) and dendritic shafts (Den) of pyramidal cells, but also at the presynaptic membrane specialization (open arrowheads) of boutons (b) of putative excitatory cells.

c, $GABA_{B1}$ and KCTD proteins are organized in clusters (arrowheads) over the surface of dendrites (Den) of CA1 pyramidal cells as assessed by the SDS-FRL technique. Insets are enlargements of the indicated frames. Scale bars (b, c) are 0.2 μm.

Modulation of G-protein signaling of $GABA_B$ receptors by KCTD proteins

To test whether coassembled KCTD proteins alter the functional properties of $GABA_B$ receptors, $GABA_{B(1,2)}$ or $GABA_{B(1,2)}$-KCTD heteromultimers were coexpressed in Xenopus oocytes or CHO cells together with the Gβγ-regulated effector ion channels Kir3 or Cav2.1/Cav2.2 (corresponding to P/Q- and N-type). Figure 5a shows typical current traces recorded in whole oocytes from heteromeric Kir3.1/3.2 channels in response to 60s-applications of 1 mM GABA to $GABA_B$ receptors assembled either from $GABA_{B1}$ and $GABA_{B2}$ ($GABA_B$) or from these core subunits and the KCTD proteins 16, 12, and 12b ($GABA_B$+KCTDx). While all receptor types effectively activated Kir3 channels, the respective K^+ currents markedly differed in their time-course: currents activated by $GABA_B$+KCTD16 and $GABA_B$+KCTD8 decreased only slightly over the application period (~10% of maximum), very similar to KCTD-less $GABA_B$ receptors, whereas the responses of both $GABA_B$+KCTD12 and $GABA_B$+KCTD12b receptors displayed pronounced desensitization (> 70% of maximum; $P < 0.001$, Dunnett's multiple comparison test, Figure 2-5a, c). The extent of the KCTD12-induced desensitization was essentially independent of the agonist-concentration in the range between 10 μM and 1 mM (rel. desensitization after 60 s was 0.72 ± 0.06 (n = 12), 0.70 ± 0.01 (n = 12) and 0.70 ± 0.08 (n = 8) for 1 mM, 100 μM and 10 μM GABA, respectively). Closer analysis showed that adequate description of the desensitization time course of $GABA_B$+KCTD12-triggered currents required two exponential functions with time constants of 4.6 ± 1.5 s (mean ± SD, n = 8; relative contribution of 0.27 ± 0.14) and 22.7 ± 4.5 s (n = 8; relative contribution of 0.73 ± 0.14; Figure 2-5c). Interestingly, KCTD12/12b-mediated desensitization also affected the 'basal Kir3 current' (activated by free $G_{\beta\gamma}$ (Peleg et al., 2002), S Figure 2-4), as visualized by its transient decrease observed upon removal of the agonist (Figure 2-5a).

The results on KCTD subtype-specific properties of $GABA_B$-receptor signaling were closely reproduced when the Kir3 channels were replaced as effector system by Cav2.1 or Cav2.2 channels. Thus, while activation of $GABA_B$, $GABA_B$+KCTD16 or $GABA_B$+KCTD8 led to stable inhibition of the Cav channels over the application period, activated $GABA_B$+KCTD12 or $GABA_B$+KCTD12b receptors decreased the Cav channel activity only transiently reflecting the pronounced receptor desensitization (~60% of maximum; Figure 2-5b, c).

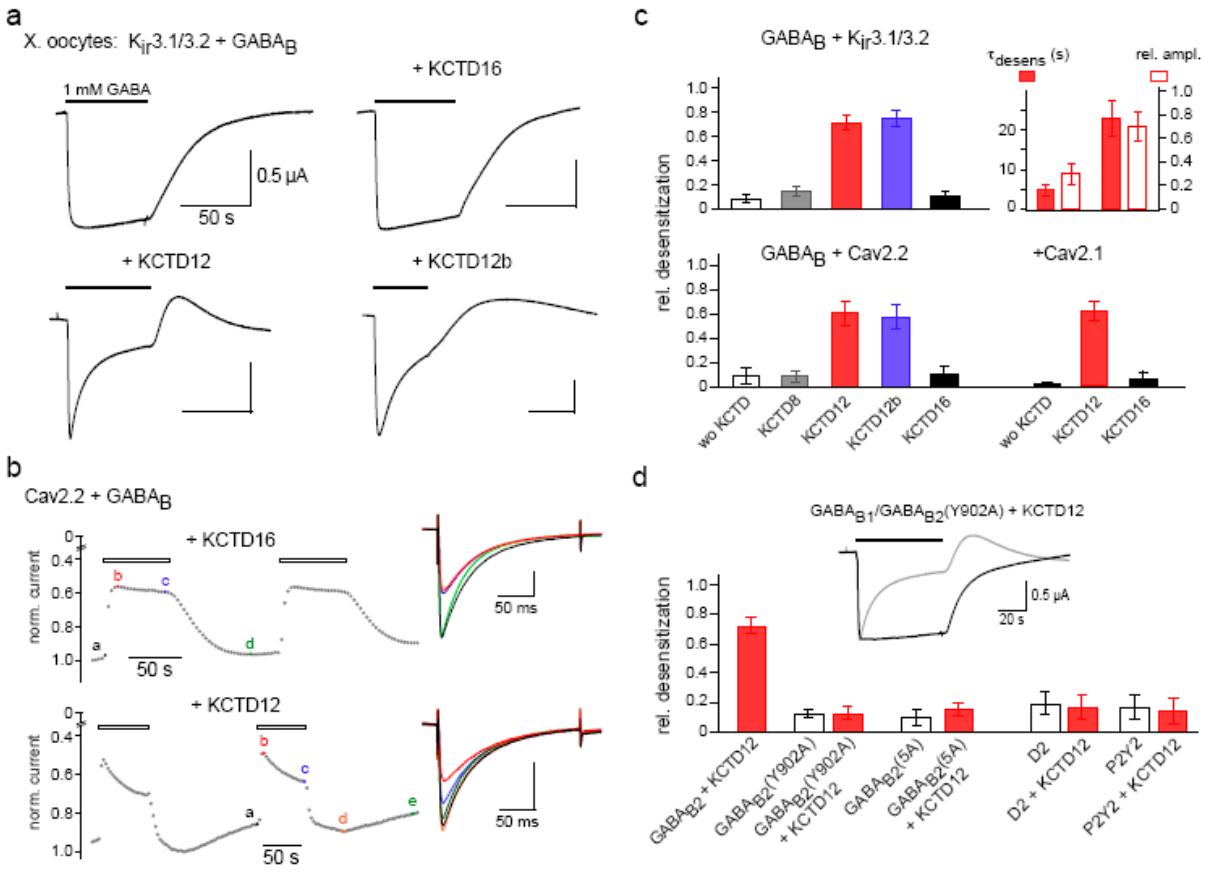


Figure 2-5: G-protein signaling of $GABA_B$ receptors is determined by KCTD proteins associated with the $GABA_{B2}$ subunit.

a, K^+ currents recorded at -50 mV in response to applications of 1 mM GABA from whole oocytes coexpressing $GABA_B$, Kir3.1/3.2 channels and the indicated KCTD proteins. Extracellular K^+ concentration was 100 mM; current and time scaling as indicated. **b**, Experiments as in (a) but with oocytes coexpressing $GABA_B$, Cav2.2 channels and the indicated KCTD proteins; the bath solution contained 10 mM Ba^{2+}. Data points are maxima of currents elicited every 3 s by voltage-steps from -100 mV to 0 mV (shown on the right for the indicated time points), successive GABA applications are denoted by the horizontal bars. Current scale is 0.1 nA, time scaling as indicated. **c**, Bar graphs summarizing the relative desensitization determined for the indicated subunit combinations. Data points are mean ± SD of 6-12 ($GABA_B$+Kir3.1/3.2), 8-13 ($GABA_B$+Cav2.2) and 3-7 ($GABA_B$+Cav2.1) experiments. Upper right panel: Time constants (τ_{desens}) and their relative contribution obtained from bi-exponential fits to the Kir3.1/3.2 current decay recorded in experiments as in (a) with KCTD12; data points are mean ± SD of 8 measurements. **d**, Summary of relative Kir3.1/3.2 desensitization determined with the indicated receptors in the absence and presence of KCTD12. Inset: Representative Kir3.1/3.2 current responses activated with $GABA_B$-KCTD12 receptors harboring either the $GABA_{B2}$ WT (grey) or the $GABA_{B2}$(Y902A) mutant subunit (black). Note that desensitization is abolished in the $GABA_{B2}$(Y902A) mutant precluding assembly with KCTD12.

The KCTD12-induced desensitization, however, was only observed if WT $GABA_{B2}$ was coassembled into the receptor complexes. Mutant $GABA_{B2}$ that precluded interaction with the KCTD proteins (Figure 2-3b), effectively prevented desensitization by the coexpressed KCTD12 protein and instead promoted G-protein signaling indistinguishable from KCTD-less $GABA_B$ receptors (Figure 2-5d). Moreover, KCTD12 failed to exert any obvious effect on the

G-protein signaling of D2 dopamine receptors and purinoreceptors of the P2Y2-subtype, although both GPCRs effectively activated the coexpressed Kir3 channels in Xenopus oocytes (Figure 2-5d).

Another set of experiments used CHO cells, because they allowed for faster solution exchange (time constant of ~12 ms) and offered a different cellular background for the receptor mediated G-protein signaling. Figure 6a shows representative traces of receptor-activated Kir3 currents, illustrating that the pronounced differences in kinetics and in extent of desensitization seen with the four KCTD subtypes in oocytes were closely recapitulated in CHO cells (at the end of a 25s-application of 0.1 mM baclofen; Figure 2-6a). In particular, approximation of the KCTD12-induced desensitization by exponential functions required two components with time constants of 1.5 ± 0.4 s (mean ± SD, n = 10; relative contribution of 0.42 ± 0.14) and 8.9 ± 2.1 s (n = 10; relative contribution of 0.58 ± 0.14; Figure 2-6a). Furthermore, rapid solution exchange was used to resolve onset and activation phases of the receptor responses. Representative Kir3 currents (Figure 2-6b, inset) indicated that all four KCTD proteins markedly accelerated the $GABA_B$ response, although to different extents. Thus, the values (mean ± SD) determined for the 20-80% rise time of the Kir3 currents revealed that KCTD8 speeded the activation by ~3-fold, while KCTD12 accelerated it by almost 10-fold (20-80% rise times of 2.90 ± 0.72 s (n = 17), 1.05 ± 0.36 s (n = 11) and 0.36 ± 0.12 s (n = 13) for $GABA_B$, $GABA_B$+KCTD8 and $GABA_B$+KCTD12 receptors, respectively; $P < 0.001$, Dunnett's multiple comparison test; Figure 2-6b). In addition to their impact on the kinetics, KCTDs 12 and 16 significantly influenced the agonist concentration-dependence of the $GABA_B$ responses as determined by apparent dose-response relationships with baclofen. As shown in Figure 2-6c, KCTD12 and KCTD16 shifted the respective EC_{50} values (fits to the mean currents) towards lower concentrations by factors of about 7 and 3, respectively.

Finally, the KCTD effects were examined in cultured hippocampal neurons that have robust Kir3-mediated $GABA_B$ responses (Sodickson and Bean, 1996). Here, recordings using fast solution exchange (time constant of ~22 ms) showed that the baclofen-elicited K^+ currents exhibited rapid onset with values for the 20-80% rise time of 0.36 ± 0.11 s (n = 26) and a steady-state desensitization of 34 ± 9% (n = 38; Figure 2-6d). Both values suggested endogenous expression of KCTD proteins that was confirmed by quantitative PCR revealing transcripts of KCTDs 8, 12 and 16 (S Figure 2-5). Upon transfection with the four KCTD isoforms, a significant increase in steady-state desensitization was only obtained with KCTDs 12 and 12b (80 ± 7% (n = 15) and 70 ± 8% (n = 7), respectively; $P < 0.001$, Dunnett's multiple comparison test; Figure 2-6d), in close agreement with the results from CHO cells and Xenopus oocytes. Moreover, KCTDs 12 and 12b also induced a significant decrease of

the 20-80% rise times (0.16 ± 0.05 s (n = 11) and 0.21 ± 0.05 s (n = 7) for KCTD 12 and 12b, respectively; P < 0.001, Dunnett's multiple comparison test; Figure 2-6d).

Taken together, these results indicated that KCTD proteins may extensively modify the kinetics of the G-protein signaling as well as the agonist-potency at $GABA_B$ receptors. The effects of the KCTD proteins are independent of the cellular background or the effector system, but require tight assembly with the receptor core, similar to the interactions between the auxiliary and the pore-forming subunits of ion channels and transporters (Arikkath and Campbell, 2003; Jentsch, 2008; Rettig et al., 1994; Schwenk et al., 2009).

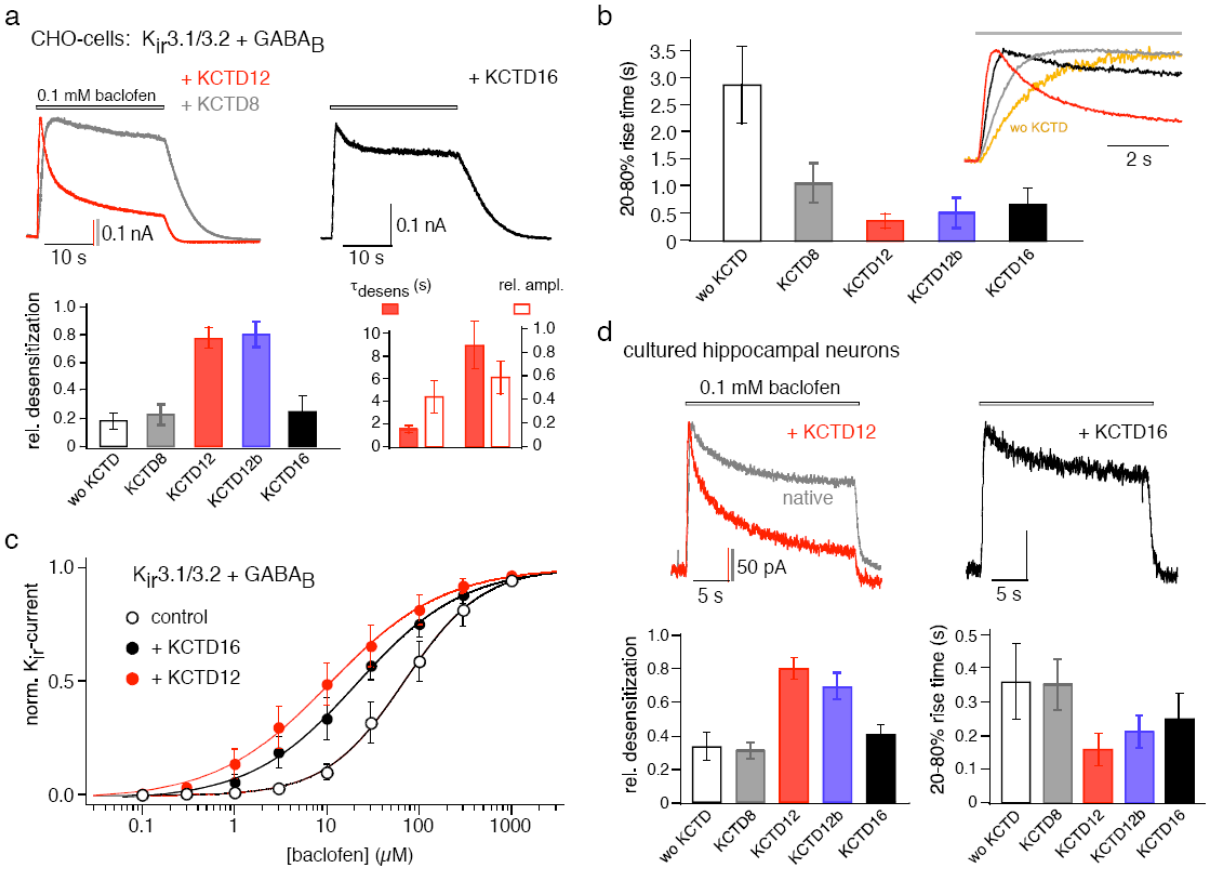


Figure 2-6: Kinetics and agonist-potency of the $GABA_B$ response is controlled by KCTDs in CHO cells and neurons.

a, Upper panel: K^+ currents through Kir3.1/3.2 channels recorded at -50 mV in response to applications of 0.1 mM baclofen from whole CHO cells coexpressing $GABA_B$, Kir3.1/3.2 channels and the indicated KCTD proteins. Extracellular K^+ concentration was 2.5 mM; current and time scaling as indicated. Lower panel: Summary plot as in Figure 2-5c; data are mean ± SD of 6-31 (relative desensitization) and 10 experiments (τ_{desens} and relative amplitude). **b**, Onset of the response given as 20-80% rise time of Kir3.1/3.2 currents activated by $GABA_B$ without or together with the indicated KCTD proteins. Data points are mean ± SD of 6-17 experiments. Inset: Representative current onsets at the indicated time scale. **c**, Apparent dose-response relations obtained with $GABA_B$ (open circles) or with $GABA_B$ and KCTD proteins 12 and 16. Data points are mean ± SD of 7-13 experiments; lines are fit of a logistic function with values for EC_{50} and Hill coefficient of 68.7 μM and 1.02 ($GABA_B$), 21.0 μM and 0.75 ($GABA_B$ +KCTD16), 9.9 μM and 0.63 ($GABA_B$+KCTD12). **d**, Upper panel: Currents through

Kir3 channels recorded in experiments as in (a) from cultured hippocampal neurons without (native) or after transfection with KCTD12 (red trace) or KCTD16; current and time scaling as indicated. Lower panels: Bar graphs summarizing relative desensitization (left) and onset (right) of the Kir3 currents as in (a) and (b); data points are mean ± SD of 7-38 experiments.

Discussion

We identified four closely related KCTD proteins as intrinsic auxiliary subunits of native $GABA_B$ receptors that are demonstrated to form high-molecular weight complexes from KCTD tetramers and the core subunits $GABA_{B1}$ and $GABA_{B2}$. The KCTD proteins endow the receptor complexes with subtype-specific properties of their G-protein signaling and provide a molecular basis for functional diversity of $GABA_B$ receptors in the mammalian brain.

KCTD proteins - novel auxiliary subunits of $GABA_B$ receptors in the brain

For investigation of the molecular composition of native $GABA_B$ receptors, we used a proteomic approach that combines APs of efficiently solubilized high-molecular weight complexes (as controlled by BN-PAGE; Figure 2-1d) from WT and knock-out brains with quantitative evaluation of nano-LC MS/MS analysis (Berkefeld et al., 2006; Schwenk et al., 2009; Zolles et al., 2009). This unbiased approach, besides isolating equimolar amounts of the expected $GABA_{B1}$ and $GABA_{B2}$ core subunits (Figure 2-2), identified the KCTD proteins 8, 12, 12b and 16 as specific partners of $GABA_B$ receptors, while it failed to retrieve previously suggested interacting proteins (Bettler et al., 2004). So far, neither of these KCTD proteins has been implicated in $GABA_B$ physiology or any other cellular process, although for KCTD12 downregulation in patients with autism has been reported (Hu et al., 2009), as well as a high-level expression in the developing cochlea and brain (Resendes et al., 2004). Further proteomic analysis showed that the KCTD proteins partner with both types of $GABA_B$ receptors, $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ (as indicated by effective copurification from $GABA_{B1a}$ -/- and $GABA_{B1b}$-/- brains), and that this partnership is exclusive among GPCRs; no other GPCRs were identified in our anti-KCTD APs, in which the $GABA_B$ core subunits were the most abundantly copurified proteins (Figure 2-2). Moreover, quantification of MS-data and native gel electrophoresis revealed that the vast majority of $GABA_B$ receptors in the mammalian brain is coassembled with the identified KCTD proteins into high molecular weight complexes (Figure 2-1d, Figure 2-2). The interaction occurs at the C-terminus of $GABA_{B2}$ that appears to bind homo- and heterotetrameric assemblies of KCTD proteins (Figure 2-2, Figure 2-3). This quaternary structure (Figure 2-3) is in line with the recently resolved structure of KCTD5 (as KCTDs 8, 12, 12b and 16 lack the residues required for pentamer formation, similar to the tetramerizing T1-domains of Kv-type K^+ channels (Dementieva et al., 2009)).

Subsequent functional analyses revealed that the KCTD proteins influence the G-protein signaling of the $GABA_B$ receptor complexes by increasing agonist sensitivity, accelerating the onset of the receptor response and determining kinetics and extent of desensitization (Figure 2-5, Figure 2-6). Therefore, the KCTD proteins engender $GABA_B$ receptors with distinct functional properties.

Molecular mechanism of KCTD action

The KCTD-mediated effects strictly required direct interaction between both $GABA_{B2}$ and KCTD (lack of effect in the $GABA_{B2}$ mutants (Y902A) and (5A); Figure 2-5d), but were independent of the cellular background (with likely different decoration of G-proteins), the effector system (Kir3, Cav2) and the type of agonist (GABA or baclofen; Figure 2-5, Figure 2-6). Based on these observations, the KCTD action may be envisaged to take place at the receptor itself, where the KCTD tetramers are positioned to interact with both the C-terminus of $GABA_{B2}$ (Figure 2-3) and the heterotrimeric G-protein at the intracellular loops 2 and 3 of this core subunit (Duthey et al., 2002). Upon agonist binding to $GABA_{B1}$, the KCTD protein may promote two distinct actions: (i) a faster initial GDP/GTP exchange at G_α leading to an accelerated activation of the effector, and (ii) a subsequent transition of the receptor into an 'inactivated state' that precludes further GDP/GTP exchange and thus leads to a decrease of the effector activity - seen as apparent desensitization. Alternatively, the subtype-specific induction of desensitization may result from an enhanced GTPase activity of $G\alpha$ induced by the KCTD tetramer, similar to the action of RGS proteins (Chuang et al., 1998; Tinker, 2006).

Implications for $GABA_B$ receptor signaling in the CNS

Under physiological conditions, activation of pre- and postsynaptic $GABA_B$ receptors is thought to be triggered by GABA either 'spilled over' from synapses upon repetitive and/or synchronous firing(Nicoll, 2004) or released from synapses in response to single action potentials (monosynaptically evoked or unitary inhibitory responses (Otis et al., 1993; Price et al., 2008; Tamas et al., 2003)). Activation by either pathway will be accentuated by the KCTDs, as they increase agonist potency and speed up receptor-effector coupling (Figure 2-6). This should be the case for both the Cav channels as the major presynaptic effector and the Kir3 channels, the predominant effector on the postsynaptic side (Figure 2-4, Figure 2-5). Under experimental conditions, $GABA_B$ receptors have often been activated by slow bath application of baclofen, which promotes extensive desensitization of KCTD12-containing receptors (Figure 2-5, Figure 2-6) and would, therefore, be expected to result in a marked underestimation of the $GABA_B$-mediated effects. Whether the $GABA_B$ effects,

however, are indeed different between neurons expressing KCTD16 and those expressing KCTD12 remains to be elucidated.

The functional significance of the identified KCTD proteins, as well as their impact on the appearance of $GABA_B$ receptor complexes are reminiscent of auxiliary subunits of ion channels and of the receptor activity-modifying proteins (RAMPs) identified for the calcitonin and calcitonin-like GPCRs (McLatchie et al., 1998). Although largely different from each other, KCTDs and RAMPs suggest that the functional properties of other GPCRs may also be affected by specific auxiliary subunits that can be identified by a proteomic approach similar to the one used here. In conclusion, our results establish the KCTD proteins 8, 12, 12b and 16 as auxiliary subunits of $GABA_B$ receptors and novel determinants of $GABA_B$-mediated signaling in the CNS.

Methods Summary

The proteomic approach including preparation of source material, APs and high-resolution mass spectrometry, as well as immuno EM and electrophysiological recordings were done as described in references (Berkefeld et al., 2006; Schwenk et al., 2009; Zolles et al., 2009). Specifications and further details are described in the Methods.

Acknowledgements

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Methods

Molecular biology

The cDNAs used were all verified by sequencing and had the following genebank accession numbers: Y10370 ($GABA_{B1}$), AJ011318 ($GABA_{B2}$), AY615967 (KCTD8), AY267461 (KCTD12), AL831725 (KCTD12b), NM_026135 (KCTD16), X57477.1 (Cav2.1), D14157.1 (Cav2.2), NM_017346.1 (Cav β1b), AF286488.1 ($\alpha 2\delta$), NM_016574.2 (D2 receptor),

L14751.1 (P2Y2 receptor); the Kir3.1/3.2 construct is as described (Wischmeyer et al., 1997).

In situ hybridizations were done with 10 μm cryosections of freshly frozen mouse tissue using digoxigenin-labeled riboprobes that were generated by transcription of cDNAs of mouse KCTDs 8, 12, 12b and 16 in sense and antisense direction followed by alkaline hydrolyzation of the probes to an average length of 200–400 bases (Schaeren-Wiemers and Gerfin-Moser, 1993).

Biochemistry

Plasma membrane-enriched protein fractions were prepared from freshly isolated rat or mouse brains and solubilised with ComplexioLyte buffers CL-75 or CL-91 (LOGOPHARM GmbH, Germany) as given in (Schwenk et al., 2009); for rat and WT mice, preparations from at least 15 individual brains were pooled, for membrane fractions of knock-out mice preparations pooled from 10 brains were used.

BN-PAGE. Two-dimensional gel electrophoresis was done as detailed in (Schwenk et al., 2009) with CL-75 used for solubilisation. For Western analysis, the blot was split into two molecular weight ranges and analysed with *anti-GABA$_{B1}$* (Engle et al., 2006) or *anti-GABA$_{B2}$* (BD Transduction Laboratories; high MW range) and *anti-KCTD12* or *anti-KCTD16* (low MW range).

Affinity purification. Solubilised membrane fractions (2 mg, CL-91) were incubated for 2 hours at 4°C with 20 μg immobilized affinity purified *anti-GABA$_{B1}$*, *anti-GABA$_{B2}$* (Chemicon/Millipore, AB5394), *anti-KCTD12* (target sequence: aa 145-166 of KCTD12 (accession AY267461); S Figure 2-3), *anti-KCTD16* (target sequence: aa 8-23 of KCTD16 (accession NM_026135); S Figure 2-3) or control IgG (Upstate/Millipore). After brief washing, bound proteins were eluted with Laemmli buffer (DTT added after elution), shortly run into SDS-PAGE gels and digested with trypsin as described (Schwenk et al., 2009). For anti-FLAG APs cleared lysates of HEK293 cells were incubated overnight at 4°C with immobilized anti-FLAG antibody (Sigma). After separation by SDS-PAGE, purified proteins were Western analyzed with the following antibodies: anti-FLAG (Sigma), anti-HA (Covance), anti-Myc (Roche) and peroxidase-coupled secondary antibodies (Amersham Biosciences).

In-vivo protein synthesis and binding assays. Distinct domains of KCTD12 and GABA$_B$B2 were expressed in *Escherichia coli* BL21(DE3) as following constructs (in modified pET16b, pET30 vectors): KCTD12-T1 (residues 27-131 of KCTD12 with a N-terminal His$_6$-tag), Tag-KCTD12-T1 (residues 27-131 of KCTD12 fused to His$_6$-GB1 protein), KCTD12-C$_{term}$ (residues 202-327 fused to His6-GB1 protein) and GABA$_{B2}$-C$_{term}$ (residues 849-940 or 745-

940 fused to His_6-GB1 protein). Proteins were purified to homogeneity by metal chelate affinity columns (Ni-Sepharose), anion-exchange (MonoQ) and size-exclusion chromatography (SEC, Superdex 200, 10/300 GL) run in Tris-buffer (20 mM Tris/HCl pH 8, 150 mM NaCl, 2 mM DTT). For binding studies, the respective purified proteins were mixed in about equimolar ratios and incubated for 30 minutes at room temperature. After addition of 0,01% Coomassie G-250 complex formation was determined by native PAGE (12%) separation.

Analytical ultracentrifugation. 0,5 mg/ml of Tag-KCTD12-T1 dissolved in 150 mM NaCl and 20 mM Tris/HCl (pH 7,4) was loaded onto a Beckman Optima-XLA (An60-Ti rotor) equipped with absorption optics. Sedimentation equilibrium runs were performed at 12.000 rpm and 15.000 rpm. The molecular masses were determined by fitting the data with a single ideal species model (Rivas et al., 1999).

Determination of stoichiometry. For determination of subunit stoichiometry in KCTD12-T1+$GABA_{B2}$-$C_{745-940}$ complexes, SEC fractions were separated by SDS-PAGE and either stained with Sypro Orange (Bio Rad) for fluorescence-based analysis of protein bands or silver stained and digested with trypsin for quantitative LC-MS/MS analysis. The molar ratio of the two subunits was obtained by (i) comparing the fluorescence signals measured for each subunit and divided by the respective molecular mass (Sypro analysis) or by (ii) quantitative MS-analysis (see below) using a purified chimeric protein (fusion of KCTD12 and $GABA_{B2}$ expressed in Xenopus oocytes) as a 1:1 reference (Schwenk et al., 2009).

Mass spectrometry

LC-MS/MS analysis. High-resolution mass spectrometry was performed as detailed in (Schwenk et al., 2009); database searches used the SwissProt database (Mammalia, release 56.8) supplemented with the TrEMBL entry Q8C7J6 for KCTD12b.

Protein quantification. Two methods, relative peak volumes (rPV) and normalized protein abundance ($abundance_{norm}$, Figure 2-2b), were used for protein quantification, both based on peak volumes (= intensity of MS signal x retention-time x m/z-width) of individual tryptic peptides (Cox and Mann, 2008; Schwenk et al., 2009). rPV values compare the amount of each protein identified in APs with the anti-$GABA_B$ and anti-KCTD antibodies from rat and mouse WT brains with the respective amount determined in APs either with IgGs (rat) or with the anti-$GABA_B$ and anti-KCTD antibodies from $GABA_B$ core subunit knock-out animals. rPV values are calculated as the median of the ratios of the six most consistent tryptic peptides of each protein (if the MS signal of a given peptide was not detected in the controls, the detection threshold of the spectrometer (3000 volume units at the settings used) served as

the denominator in rPV calculations). Any protein with an rPV value >10 (APs from rat) or >5 (APs from mouse) were regarded specifically purified by the respective antibody.

$Abundance_{norm}$ values compare the molar amounts of different proteins and are calculated as the sum of all assigned peak volumes divided by the number of MS-accessible amino acids (sequence of tryptic peptides with masses between 740 and 3000 Da under the MS settings used). For KCTD12b, the $abundance_{norm}$ value had to be corrected to account for the significant number of peptides shared with the markedly more abundant KCTD12; the actual contribution of KCTD12b was estimated from the mean peak volume ratio of KCTD12b-specific peptides versus KCTD12-specific peptides.

MS-based subunit stoichiometry in KCTD12-T1+$GABA_{B2}$-$C_{745-940}$ complexes was determined as the median of the ratios calculated with the 4 top-ranked and calibrated (reference protein described above) peptides of $GABA_{B2}$-$C_{745-940}$ and the 5 top-ranked and calibrated peptides of KCTD12-T1 (a total of 20 ratios).

Sequence coverage. Sequence coverages (SC) of $GABA_{B1a}$, $GABA_{B2}$ and the KCTD proteins were calculated as $SC = N_i / (N_i + N_{an})$, where Ni is the number of amino acid residues of the identified peptides (with Mascot Score ≥ 20) and N_{an} is the number of MS-accessible but not identified amino acids (see above) of the respective sequence.

Electron microscopy

Pre-embedding immunoelectron microscopy. Hippocampal sections of adult Wistar rats (n = 8) were treated for single immunogold labeling with affinity-purified anti-$GABA_{B1}$, *anti-KCTD12* and *anti-KCTD16* as described (Kulik et al., 2003); the secondary antibody was coupled with 1.4 nm gold particles, signal enhancement was done with a HQ Silver kit (Nanogold; Nanoprobes, Stony Brook, NY).

SDS-digested freeze-fracture replica immunolabeling (SDS-FRL). Adult Wistar rats (n = 6) were processed for immunolabeling as described (Masugi-Tokita et al., 2007). After digestion in 2.5% SDS solution replicas were incubated first with anti-$GABA_{B1}$, *anti-KCTD12*, or *anti-KCTD16* and subsequently with a 10 nm gold-coupled secondary antibody (1.30; British Biocell International, Cardiff, UK).

Electrophysiological recordings and data analysis

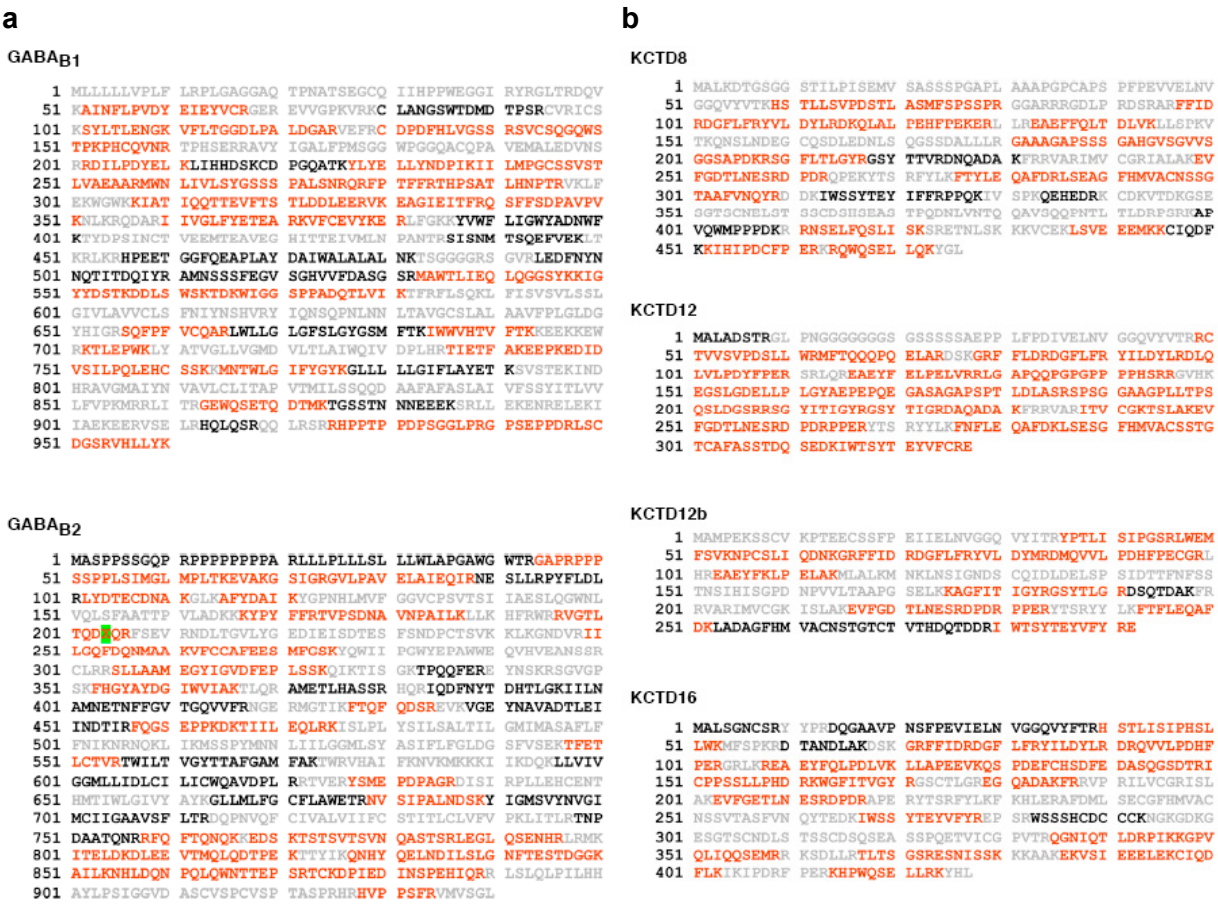
Electrophysiological recordings from whole oocytes were performed at room temperature (22-24 °C) as described previously (Schwenk et al., 2009; Zolles et al., 2006). Currents were low-pass filtered at 1 kHz, and sampled at 2 kHz; capacitive transients were compensated using the compensation unit of the amplifier (TurboTec 10CX; npi, Tamm, Germany) and a

P/4 protocol. Extracellular solution was composed as follows (mM): 100 KCl, 17.5 NaCl, 10 HEPES, and 1.8 $CaCl_2$ (pH 7.3) for Kir 3.1/3.2 channels and 3 KCl, 102 NaCl, 10 HEPES, and 10 $BaCl_2$ (pH 7.3) for Cav channels, respectively. Solution exchange time was within ~1s.

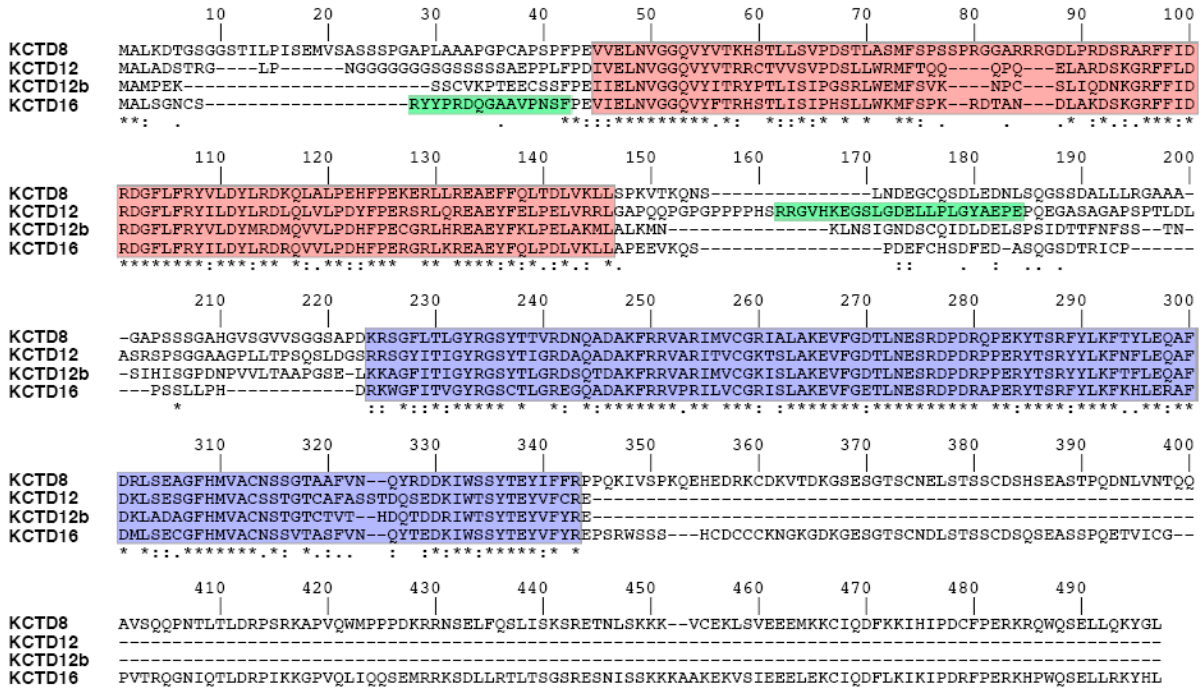
Experiments on CHO cells stably expressing $GABA_B$ and cultured hippocampal neurons were performed at room temperature (23-24° C) 1-3 days or 1-2 weeks (DIV14-21) after a transfection, respectively. Cells were continuously superfused with an extracellular solution (ECS) composed of (in mM): 145 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 25 Glucose; pH 7.3, 323 mosm. Neurons were superfused with ECS supplemented with 5 μM DNQX, 0.5 μM TTX, 0.3 μM strychnine, 100 μM picrotoxin, 5 μM D-CPP. Patch pipettes had resistances between 3-4 MΩ when filled with intracellular solution composed of (in mM) 107.5 potassium gluconate, 32.5 KCl, 10 HEPES, 5 EGTA, 4 MgATP, 0.6 NaGTP, 10 Tris phosphocreatine; pH 7.2, 297 mosm. Series resistance (< 5 MΩ) was compensated by 80%. $GABA_B$ responses were evoked by fast application of baclofen (Dittert et al., 2006) and recorded with an Axopatch 200B patch-clamp amplifier; filtering and sampling frequencies were set to 1 kHz and 5 kHz, respectively.

Relative desensitization was calculated as the reduction in effect (increase for Kir currents, decrease for Cav currents) measured during prolonged agonist applications (60s or 25s): 1-(agonist-induced effect at the end of application / maximal agonist-induced effect) x 100%. Desensitization time constants were derived from bi-exponential fits to the decay phase of Kir3.1/3.2 currents during agonist application. Curve fitting and further data analysis was done with Igor Pro 4.05A Carbon; data are given as mean ± SD/SEM as indicated in text and figures legends. Statistical significance was assessed using ANOVA with the Dunnett's multiple comparison test.

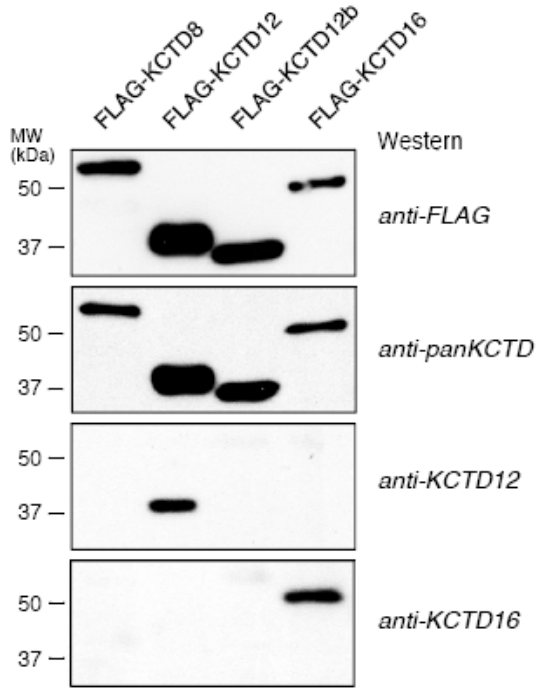
Supplemental figures



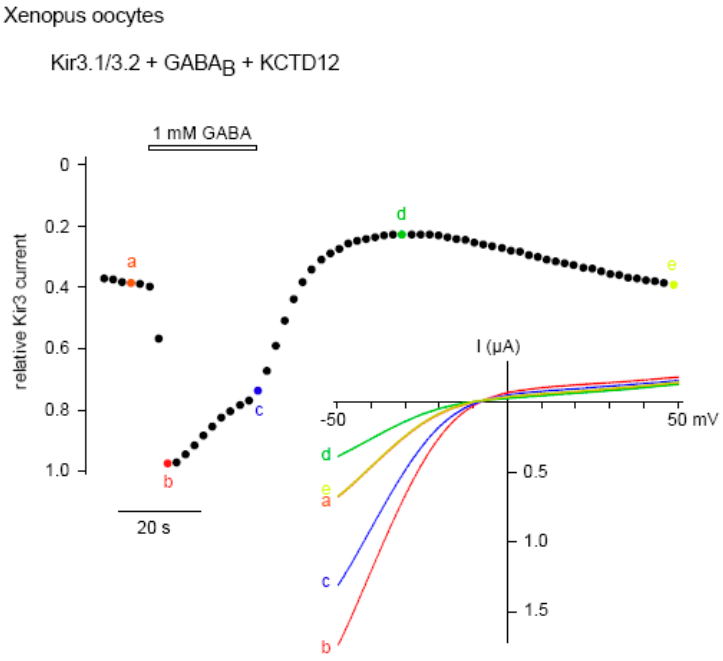
S Figure 2-1: Primary sequences of $GABA_{B1a}$, $GABA_{B2}$ (a) and the KCTD proteins 8, 12, 12b and 16 (b). Peptides identified by mass spectrometry are in red; those accessible to but not identified in MS/MS analyses are in black, and peptides not accessible to the MS/MS analyses used are given in grey. The $GABA_{B2}$ residue highlighted in green was identified as valine or arginine (polymorphism). The genebank accession numbers of the indicated proteins were: NP_112290.2 ($GABA_{B1a}$), NP_113990.1 ($GABA_{B2}$), NP_001093642.1 (KCTD8), EDM02446.1 (KCTD12), XP_001065910.1 (and FT174274.1, KCTD12b), XP_225971.4 (KCTD16).



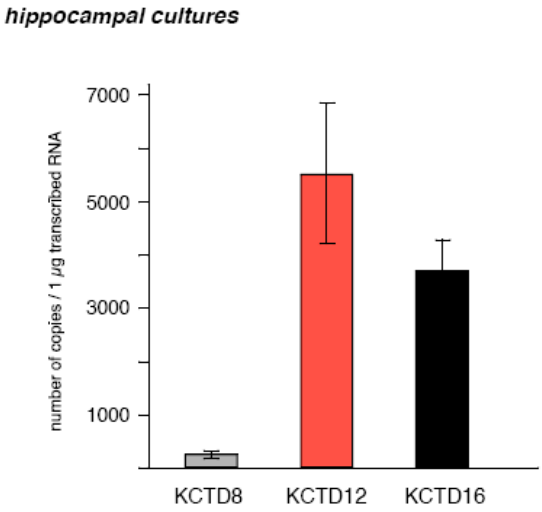
S Figure 2-2: Primary sequence alignment of the indicated KCTD proteins as obtained with the Clustal method. Regions highlighted in red and blue denote the conserved T1- and C-terminal domains, respectively; the green boxes highlight the epitopes used for the generation of *anti-KCTD12* and *anti-KCTD16*.



S Figure 2-3: Evaluation of subtype-specificities of the *anti-KCTD12* and *anti-KCTD16* antibodies. SDS-PAGE separated lysates of HEK293 cells expressing the indicated FLAG-tagged KCTD proteins were Western probed with anti-FLAG, *anti-KCTD12* and *anti-KCTD16*. An antibody targeting KCTD isoforms 8, 12, 12b and 16 (*anti-panKCTD*) was added for comparison.



S Figure 2-4: Basal (prior to and after agonist application) and GABA-activated Kir3 currents recorded in oocytes coexpressing $GABA_B$ and KCTD12 together with Kir3.1/3.2 channels. Currents were elicited by voltage ramps from -50 to 50 mV (2s duration) and the maxima (at -50 mV) were plotted over time. Current traces shown on the right were recorded at the indicated time points. Note that both basal and agonist-triggered currents are mediated by Kir3.1/3.2 channels.



S Figure 2-5: Expression analysis of the indicated KCTD proteins in cultured hippocampal neurons by quantitative PCR. Data are mean ± SD of 3 different cultures. qRT-PCR was carried out using the Roche LightCycler with the FastStart DNA Master SybrGreen (Roche, Basel, Switzerland); the primer sequences used were:
mKCTD8 (5′-CAACCAGTACCGAGACGACA-3′) and (5′-TGTTCTTGCTTGGGT-GACACT-3′); mKCTD12 (5′-AGACATTTCTGTACCTTCCACCA-3′) and (5′-TTA-TACTAATGGCCCAATCTTTAACA-3′); mKCTD16 (5′-GGTCGAGCTATACTGAG-TACGTCTT-3′) and (5′-TCTTGCAGCAGCAGTCACA-3′); 60s: (5′-GGAAGTA-CCAGGCAGTGACAG -3′) and (5′-GCAGGCATGAGGCAAACAG-3′). cDNA copy numbers for each gene were quantified by using standard curves of known quantities of plasmids containing the corresponding PCR products. 60s ribosomal protein L13A was used for normalization.

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Personal contribution

Design of experiments

Construction of KCTD and $GABA_{B2}$ mutants plasmids used in Co-IP, oocyte experiments

Interaction study of KCTD $GABA_B$ receptor

Mapping of the KCTD binding site on $GABA_{B2}$

Generation and characterization of antibodies used in reverse APs, EM

ISH analysis

RT-PCR

Design of figures

3 The C-terminus of KCTD12 promotes desensitization of $GABA_B$ receptors in neurons

(in preparation)

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Abstract

$GABA_B$ receptors are the G-protein-coupled receptors (GPCRs) for gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. $GABA_B$ receptors are obligate heterodimers, and as such considered a prototypic receptor for many GPCRs. Recently, a functional proteomics approach identified a family of potassium channel tetramerization domain containing (KCTD) proteins as auxiliary subunits of native $GABA_B$ receptors. These proteins, KCTD 8, 12, and 16 are intrinsic components of high molecular weight $GABA_B$ receptor complexes isolated from synaptic plasma membranes and where shown to determine the functional properties of $GABA_B$ receptors in heterologous cells and neurons. In this report, we demonstrate that the multi-domain organization of these auxiliary subunits provide the means for the assembly of high molecular weight $GABA_B$ receptor complexes. Structure function analysis shows that the N-terminal tetramerization domain of the KCTD proteins binds to the $GABA_B$ receptor, whereas the C-terminal conserved domain is involved in homo- and heteromerization. Moreover, we identified the C-terminal domain of KCTD12 as the critical determinant for the modulation of $GABA_B$ receptor desensitization in organotypic hippocampal slice cultures. Finally, two-photon scanning microscope analysis of the neuronal distribution of the $GABA_B$ receptor auxiliary subunits illustrates a differential distribution in pre- and postsynaptic structures. This indicates that the composition of native $GABA_B$ receptors is differentially regulated depending on their subcellular localization in neurons. Taken together, our findings demonstrate the complex nature of the novel $GABA_B$ receptor auxiliary subunits, which may underlie the functional heterogeneity of native $GABA_B$ receptors.

Introduction

$GABA_B$ receptors are the G-protein coupled receptors (GPCRs) for gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. $GABA_B$ receptors exist as obligate heterodimers, comprising the $GABA_{B1}$ subunit which binds the agonist GABA and the $GABA_{B2}$ subunit which is essential for surface trafficking of the receptor and mediates G-protein coupling (Jones et al., 1998). Two isoforms of the $GABA_{B1}$ subunit exist: $GABA_{B1a}$ and $GABA_{B1b}$, that differ in a pair of sushi repeats present only in the extracellular N-terminus of $GABA_{B1a}$ (Kaupmann et al., 1997, 1998; White et al., 1998). Presynaptic and postsynaptic $GABA_B$ receptors regulate Ca^{2+}- and K^+-channels, respectively, via the $G\beta\gamma$ subunits. Furthermore, $GABA_B$ receptors can activate and inhibit adenylyl cyclase via the $G\alpha i/o$ and $G\beta\gamma$ subunits. Postsynaptically, $GABA_B$ receptors couple to Kir3-type K^+ channels hyperpolarizing the postsynaptic membrane (Kaupmann et al., 1998). Presynaptically,

$GABA_B$ receptors inhibit Ca^{2+} channels leading to decreased neurotransmitter release. Thus they regulate excitatory and inhibitory neurotransmission (reviewed by Bettler et al., 2004). Previous studies have sought explanations for the functional heterogeneity of native $GABA_B$ receptors by isolating protein interactors and examining the functional consequences (reviewed in Tiao and Bettler, 2006). Despite the identification of numerous $GABA_B$ receptor interactors from the intracellular milieu, protein interactions isolated within the context of a native environment remains a challenge. Recently, a class of novel $GABA_B$ receptor interacting proteins, KCTD8, 12, 12b, and 16, were discovered using a proteomics approach, and were proposed to act as auxiliary $GABA_B$ receptor subunits altering receptor functions (Schwenk et al., 2009). It was shown that these proteins bind specifically to the intracellular region of the $GABA_{B2}$ subunit. These novel $GABA_B$ receptors interacting proteins belong to the K^+-channel tetramerization domain (KCTD) containing protein family whose members characteristically contain an N-terminal tetramerization (T1) domain that is homologous to the T1 domain of some voltage-gated K^+ (Kv) channels. Apart from this domain, the four $GABA_BR$ interacting KCTDs are phylogenetically distinct from the other family members; KCTD8, 12, 12b, and 16 also share high sequence homology within their C-termini, thus forming a KCTD subfamily.

A study by Schwenk et al. has shown that KCTD8, 12, and 16 are tightly associated members of native $GABA_B$ receptor complexes, and moreover that these $GABA_B$ interacting proteins alter $GABA_B$ receptor mediated functions (Schwenk et al., 2009). In the present study we identify the structural determinants of these proteins which are required for interactions among the KCTDs, for the interaction with the $GABA_{B2}$ subunit, and as well for mediating changes in $GABA_B$ receptor function. We show that these proteins interact with each other in vitro and in vivo, and their ability to form homo- and heteromers. We found that KCTD8, 12 and 16, unlike other members of the KCTD protein family, oligomerize through their C-termini. Additionally we demonstrate that the T1 domain is responsible for interaction with the $GABA_{B2}$ subunit. The $GABA_B$ interacting KCTDs have been shown to alter $GABA_B$ receptor function (Schwenk et al., 2009). Stimulation of $GABA_B$ receptors followed by G-protein activation leads, within milliseconds, to the opening of Kir3-type K^+ channels. With persistent stimulation of the GPCR the amplitude of current may decrease with time. This reduction in current level is referred to as ‘‘desensitization’’ and represents an adaptive response of the cell to prevent excessive G-protein signaling. Synaptic activation of $GABA_B$ receptors in the dendrites of neurons produces a slow inhibitory postsynaptic potential (IPSP) that is carried by Kir3-type K^+ channels (Sodickson et al., 1996). Postsynaptic $GABA_B$ receptors are postulated to be located extrasynaptically, requiring elevated levels of synaptic-released GABA, such as might occur during stimulation of multiple interneurons (Scanziani et

al., 2000). During such sustained activation, $GABA_B$ receptors may undergo desensitization. In our study we examined the effects of KCTD8, 12, and 16 on the kinetics of $GABA_B$-mediated Kir3-type K^+ currents and assessed whether KCTD8, 12, and 16 induce or modulate desensitization of K^+ currents. We aimed to work under conditions in which the physiological neuronal networks are highly preserved, and therefore chose hippocampal organotypic slice cultures as experimental system. We found that in this system KCTD12 induces desensitization of $GABA_B$-mediated K^+ currents and that the C-terminus of KCTD12 is necessary for this effect. Finally, we characterized the subcellular distribution of KCTD8, 12 and 16. While all three proteins are distributed throughout the cytosol of the neuron, differential distributions could be observed in axonal and in dendritic compartments. The ability of the $GABA_B$ interacting KCTDs to form heteromeric complexes combined with the different effects of the individual KCTDs on $GABA_B$ receptor function and their differential distribution in neuronal compartments may provide one possible mechanism to constitute different subtypes of $GABA_B$ receptor complexes which differentially regulate physiological fine tuning of the $GABA_B$ response.

Results

KCTD8, 12, and 16 form homomeric and heteromeric complexes

The T1-domain of Shaker K^+-channels is known to interact with other T1-domains, albeit the exact function of this domain remains controversial (Shen and Pfaffinger 1995; Kobertz and Miller 1990, Li et al 1992; Sewing et al. 1996; Gulbis et al., 2000). It was therefore proposed that KCTD proteins are capable of forming homo- and hetero-oligomers. To this end, we expressed KCTD proteins in HEK293 cells to examine the interaction among KCTD8, 12, and 16. Indeed, co-immunoprecipitation experiments with FLAG- and Myc-tagged KCTD proteins show that KCTD8, 12, and 16 can form homomeric (Figure 3-1A) as well as heteromeric complexes (Figure 3-1B). For quantification, proteins were also expressed in the yeast-two-hybrid system and results corroborate with the co-immunoprecipitation data (Figure 3-1A). Furthermore, the quantitative data indicates a very strong homomeric interaction between KCTD12 molecules, followed by a weaker interaction of KCTD8 homodimers. Likewise, KCTD12 forms the strongest heterodimeric interactions followed by KCTD8. The non-$GABA_{B2}$ interactor KCTD10 forms a strong homomeric complex, but does not interact with KCTD8, 12 or 16 (S Figure 3-1B). In the yeast, interactions involving KCTD16 are consistently weak, likely due to an incompatibility of the GAD-KCTD16 fusion protein with the yeast two-hybrid system (S Figure 3-1A and B). To confirm homo- and hetero-dimerization of endogenous KCTD proteins, mouse whole brain cytosolic lysates were

prepared, KCTD proteins were immunoprecipitated with antibodies specific for KCTD8, 12, and 16 and co-immunoprecipitated proteins were visualized on Western blots. Co-immunoprecipitation of endogenous proteins shows that all KCTD proteins examined co-precipitate with each other (Figure 3-1C), which confirms the data obtained in heterologous systems.

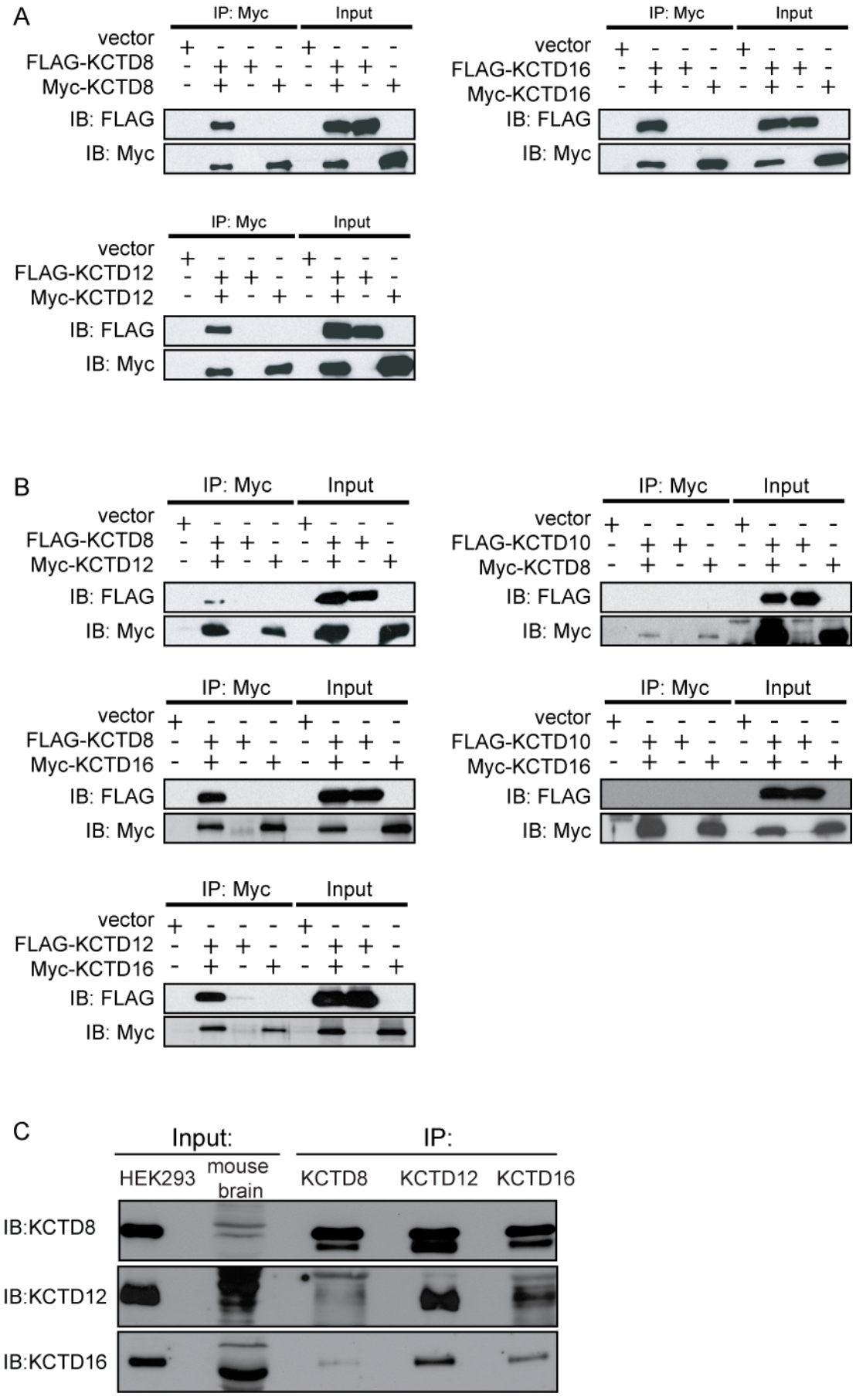
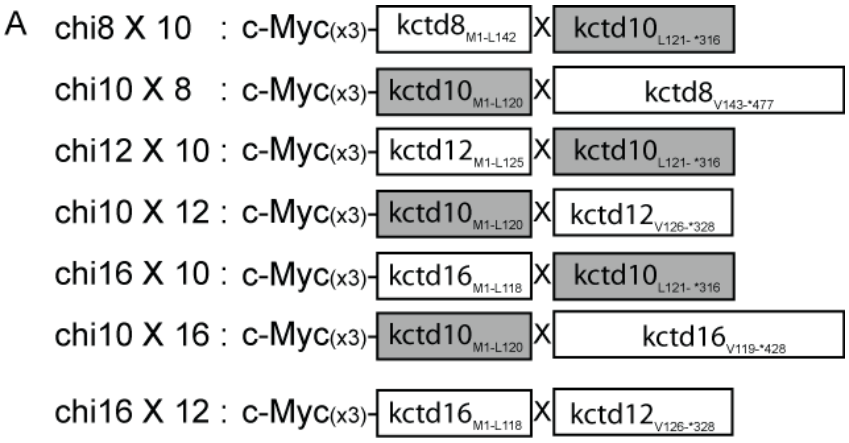


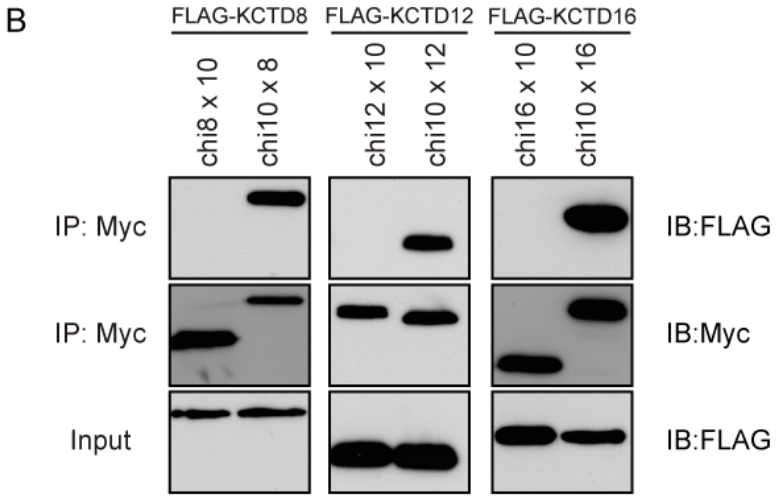
Figure 3-1: KCTD8, 12, and 16 form homo- and heterodimers.

(A) KCTD homodimerization and **(B)** heterodimerization: Cell lysates of HEK293 cells co-expressing differentially epitope tagged wild-type KCTD8, 12, and 16 were immunoprecipitated with anti-c-Myc antibody, separated by SDS-PAGE and immunoblotted with anti-FLAG antibody. Total immunoprecipitated c-Myc epitope tagged protein were immunoblotted using anti-c-Myc antibody. **(C)** Endogenous KCTD8, 12, and 16 protein homo- and heterodimerization. Wild-type mouse KCTDs were immunoprecipitated from cytosolic brain protein lysates with specific rabbit anti-KCTD8, 12, and 16 antibodies and visualized by immunoblotting using guinea-pig anti-KCTD8, 12, and 16 specific antibodies. Representative blots of at least three independent experiments are shown.

KCTD complex formation is mediated via the C-terminus

As the KCTDs can be roughly divided into two parts, the N-terminus, containing the T1 domain and the C-terminus containing a domain that is conserved within the $GABA_B$ interacting KCTDs, joined by a short peptide linker (S Figure 3-2A), we used molecular means to isolate the domain critical for homo- or heteromeric complex formation. To mimic a full-length protein conformation, we generated KCTD chimeras composed of either the N-terminus (including the conserved T1 domain) of KCTD8, 12 or 16 fused to the C-terminus (peptide sequences C-terminal of the T1-domain) of the non-interacting KCTD10 or the N-terminus (including the conserved T1 domain) of the non-interacting KCTD10 fused to the C-terminus (peptide sequences C-terminal of the T1 domain) of KCTD8, 12 or 16 (Figure 3-2A). We took advantage of KCTD10, as its structure is similar to KCTD8, 12, and 16 but it does neither bind to the $GABA_B$ receptor nor to one of the $GABA_B$ interacting KCTDs. The chimeric proteins were co-expressed in heterologous cells and protein interaction identified using co-immunoprecipitation. The results demonstrate that the C-terminus of KCTD8, 12, and 16 contains the pre-requisite peptide residues critical for homodimerization (Figure 3-2B) and heterodimerization (Figure 3-2C). Unlike previous postulations concerning the role of T1-domains in Shaker K^+-channel protein interaction, the T1 domain of KCTD8, 12, and 16 is not required for homo- and/or hetero-dimerization. Rather, in KCTD8, 12, and 16 the C-termini are essential for the dimerization of KCTD proteins.





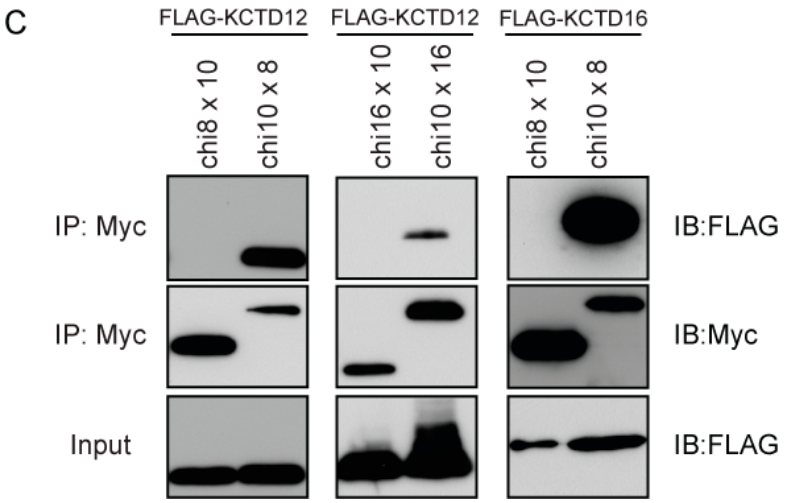


Figure 3-2: The C-terminus of KCTD8, 12, and 16 confers protein interaction.
(A) Schematic of recombinant chimeric-proteins generated for mapping KCTD8, 12, and 16 dimerization domain. To map the homodimerization domain **(B)** and the heterodimerization domain **(C)**, KCTD chimeric (c-Myc tagged) and wild-type (FLAG tagged) proteins were co-expressed in HEK293 cells. For co-immunoprecipitation, cell lysates were incubated with anti-Myc antibody and immunoblotted using anti-FLAG antibody. Representative blots of at least three independent experiments are shown.

Interaction with $GABA_{B2}$ is mediated via the T1-domain

Previously, it was shown that KCTDs bind specifically with the extreme C-terminal domain of the $GABA_{B2}$ subunit (Schwenk et al., 2009). It remains, however, unidentified which KCTD domain is critical for $GABA_{B2}$ binding. To decipher the region in KCTD8, 12, and 16 that is responsible for interaction, we used chimeric proteins described above. In co-immunoprecipitation experiments, chimeric proteins containing either the KCTD12 (chi12x10) and/or KCTD16 (chi16x10) T1-domain were able to maintain interaction with $GABA_{B2}$ (Figure 3-3). In contrast, chimeric proteins lacking the T1-domain of KCTD12 (chi10x12) or KCTD16 (chi10x16) were unable to interact with $GABA_{B2}$. Interestingly, similar to the negative KCTD10 control, both chi8x10 and chi10x8 chimeric proteins were unable to interact with $GABA_{B2}$. Based on these results, the interaction of KCTD12 and KCTD16 with $GABA_{B2}$ is mediated via the T1 domain, whereas both the T1 as well as the C-terminus of KCTD8 are necessary for $GABA_{B2}$ interaction.

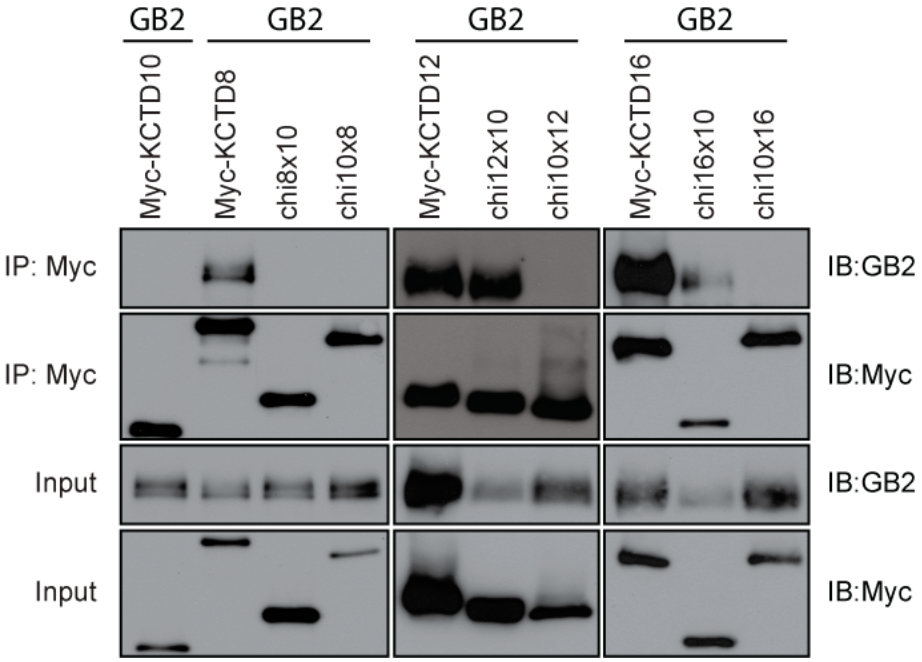


Figure 3-3: The T1-domain in KCTD8, 12, and 16 is critical for the interaction with the $GABA_{B2}$ subunit.
To isolate the $GABA_{B2}$ interacting domain, KCTD chimeric proteins were co-expressed with $GABA_{B2}$ (GB2) in HEK293. For co-immunoprecipitation, cell lysates were incubated with a rabbit anti-Myc antibody and immunoblotted using a mouse anti-Myc and an anti-GB2 antibody. Representative blots of at least three independent experiments are shown.

KCTD8, 12, and 16 are differentially distributed throughout the neuron

Previously, qualitative data using electron microscopy revealed co-localization of KCTD12 and KCTD16 with pre- and post-synaptic $GABA_B$ receptors (Schwenk et al 2009). To quantify

the subcellular distribution of KCTD8, 12, and 16 in neuronal structures, we co-expressed the individual KCTDs tagged with a green fluorescent protein (GFP) tag at the N-terminus and an ubiquitously distributed tdimer2-RFP protein (gift from R. Tsien) in organotypic hippocampal slice cultures. GFP alone was used as control. We imaged CA1 pyramidal neurons using a custom build two-photon laser scanning microscope. We found all three KCTDs distributed throughout the cytosol of hippocampal CA1 neurons (S Figure 3-3). Subsequently we focused on four main neuronal structures; dendrites, spines, axons and boutons (Figure 3-4A). After normalizing to the dendrite, GFP-KCTD8, GFP-KCTD12 and GFP-KCTD16 showed significant accumulation in the dendritic spines (Figure 3-4B). In addition, GFP-KCTD16 was also enriched significantly in boutons (Figure 3-4C). All three KCTD proteins studied displayed uniform distribution in the axon (Figure 3-4D). Different from the known nuclear KCTD family members including KCTD1 (Ding et al., 2009), KCTD10 (Wang et al., 2009) and KCTD13 (He et al., 2001), the $GABA_B$ receptor interacting KCTDs are not present in the nucleus (S Figure 3-3). As the KCTD proteins are localized throughout the cytosol and the main neuronal compartments, this places them in the same locality as the cytosolic side of pre- and postsynaptic $GABA_B$ receptors, suggesting a functional relationship between the two proteins.

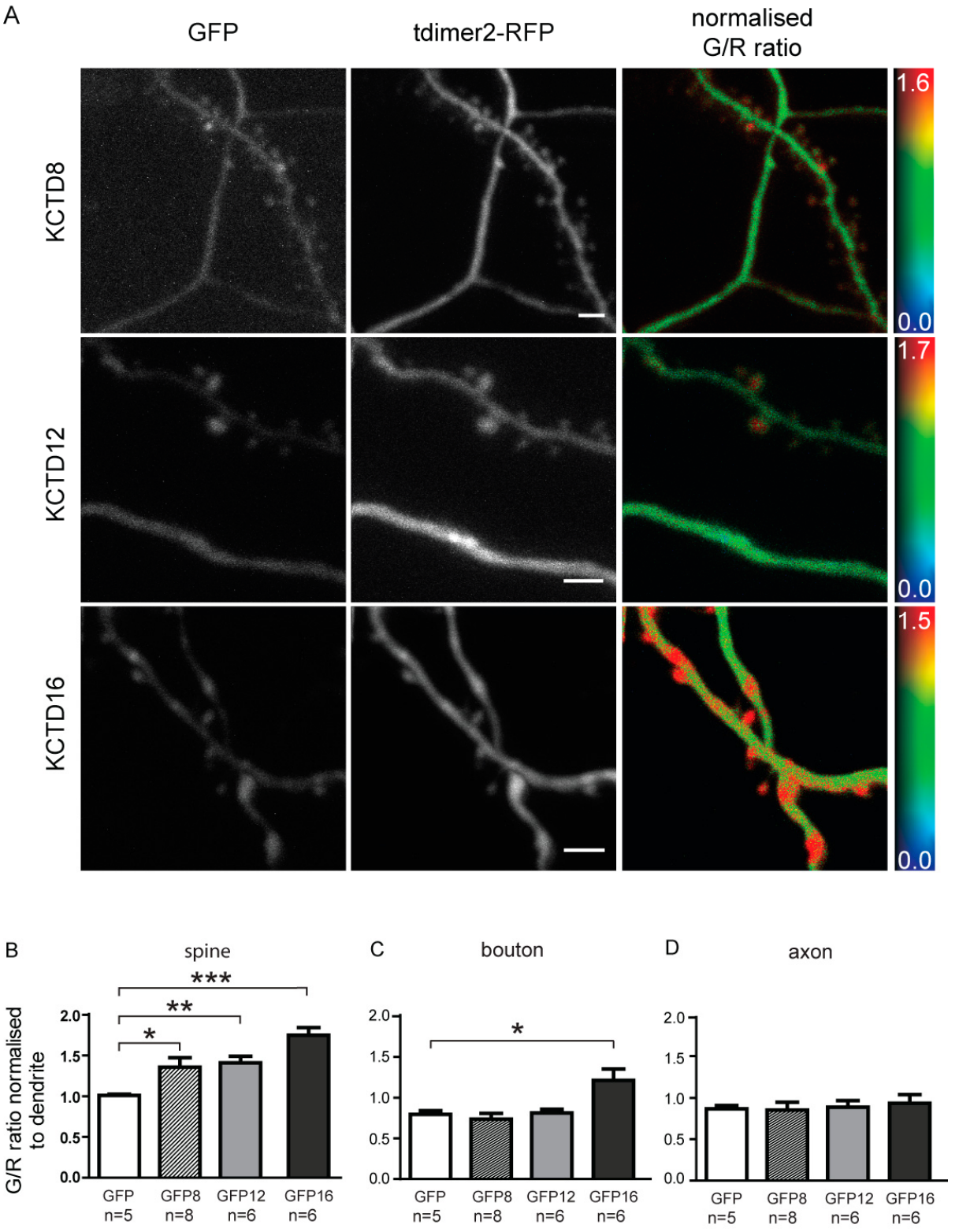


Figure 3-4: KCTD8, 12, and 16 are differentially distributed in hippocampal CA1 pyramidal neurons.

Expression of GFP-tagged KCTD8, 12, and 16 in hippocampal organotypic slice cultures. Maximum intensity projections of dendrites and axons in the CA1 region of the hippocampus expressing GFP-KCTD8, GFP-KCTD12 or GFP-KCTD16 **(A)** in combination with the freely diffusible tdimer2 RFP are shown. The ratio of green-to-red fluorescence (G/R) is coded in rainbow color. Scale bar, 2 μm. The spine, bouton, and axonal expression levels of GFP-tagged KCTDs were each normalized to the

dendritic expression level **(A)**, and distribution compared with ubiquitously expressed GFP. All the GFP-tagged KCTD proteins show significant enrichment in dendritic spines **(B)**. Predominantly GFP-KCTD16 is accumulated in the bouton **(C)** and all proteins are evenly distributed in the axon **(D)**. n = number of individual neurons imaged, all statistics are one-way ANOVA.

KCTD8, 12, and 16 differentially modulate $GABA_B$ receptor-mediated desensitization

Previously, KCTD-mediated $GABA_B$ receptor desensitization was analyzed in heterologous cell lines, Xenopus oocytes and dissociated neurons (Schwenk et al 2009). This study hypothesized that exogenous expression of KCTD12 protein in CA1 pyramidal neurons can also promote $GABA_B$ receptor desensitization. To investigate this phenomenon in an intact tissue and neuronal cell-type specific paradigm, we used patch-clamp recording to record responses in transfected CA1 pyramidal neurons in hippocampal organotypic slices.

In non-transfected CA1 pyramidal neurons, 40 s pressure application of 100 μM GABA induced an outward Kir3-type K^+ current that weakly desensitized (Figure 3-5A). Bath application of $GABA_B$ receptor antagonist CGP54626A abolished the current indicating that GABA selectively activated $GABA_B$ receptors in our conditions (Figure 3-5A and C). Overexpression of KCTD8 and KCTD16 had no effects on $GABA_B$ response kinetics and amplitudes (Figure 3-5A and C). In contrast, in neurons overexpressing KCTD12 GABA-induced currents desensitized substantially during 40 s of continuous agonist application without effects on amplitudes (Figure 3-5A and C). Activation of A1 receptors with adenosine (100 μM, 40 s), which converge on the same Kir3-type K^+ channels (Lüscher et al., 1997), induced a non-desensitizing current in non-transfected and KCTD12 overexpressed cells. Adenosine-mediated currents were abolished in the presence of A1 receptors antagonist DPCPX (Figure 3-5B and C). This indicates that the desensitization of $GABA_B$ currents mediated by KCTD12 is not a consequence of functional alterations in Kir3-type K^+ channels. Despite the fact that KCTD8, 12, and 16 bind to $GABA_B$ receptors, our results show that only KCTD12 induces desensitization of the receptor in CA1 pyramidal neurons.

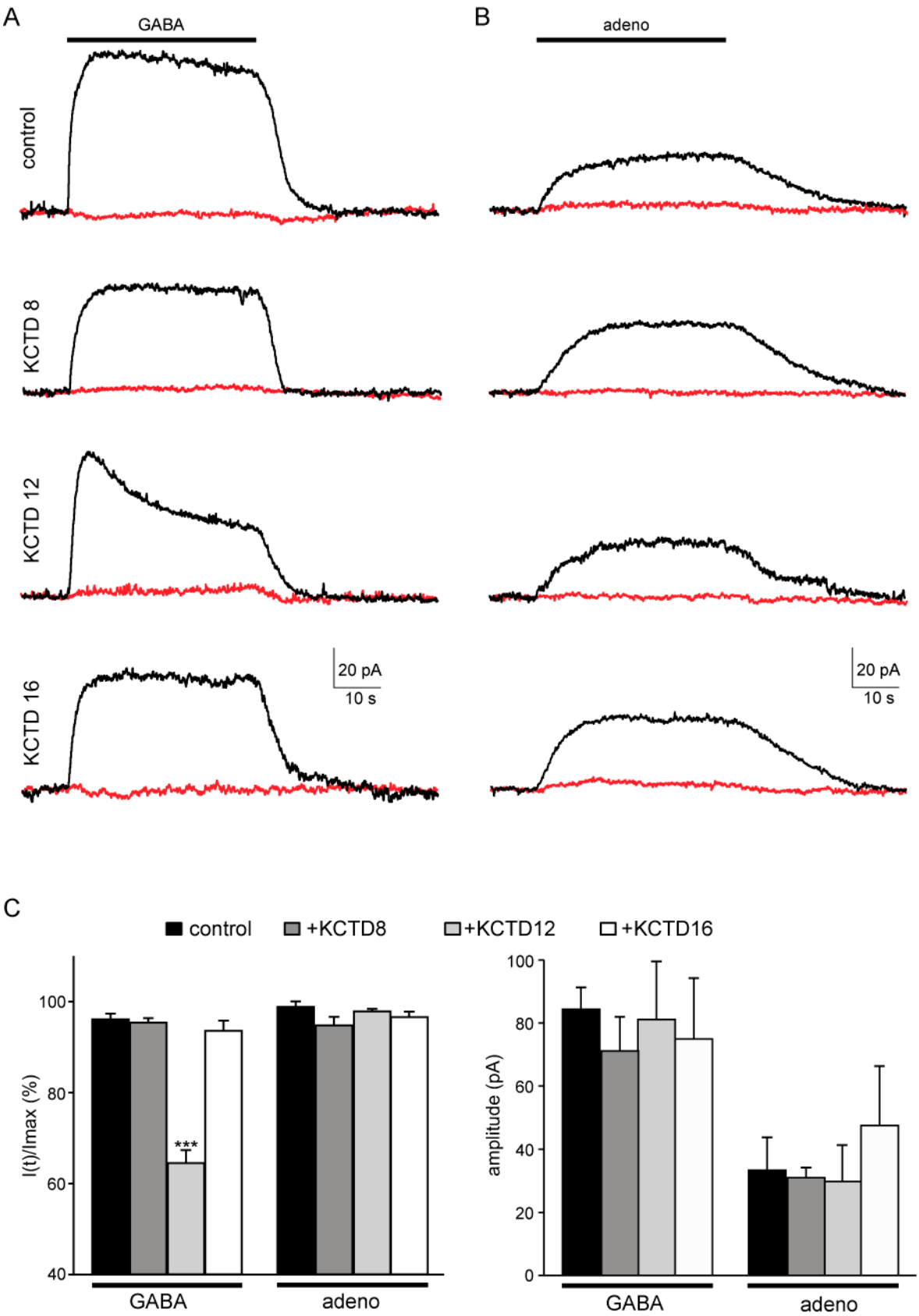


Figure 3-5: KCTD12 induces desensitization of $GABA_B$ receptors in transfected CA1 hippocampal pyramidal neurons

(A) Representative Kir3-type K^+ currents induced by pressure application of GABA (100 μM, 40s) in control or neurons transfected with KCTD8, 12 or 16. In neurons overexpressing KCTD12, $GABA_B$

responses showed an increased desensitization during agonist application (indicated above the current trace) when compared with non-transfected neurons (control) or neurons transfected with KCTD8 or KCTD16. GABA-induced outward K^+ currents were blocked by bath application of CGP 54626A (5 μM, red traces).
(B) K^+ currents induced by A1 receptors activation had similar kinetics in all conditions, suggesting that effector K^+ channels are not altered by KCTD proteins. Adenosine-induced currents were blocked by bath application of the antagonist DPCPX (1 μM, red traces). Currents were recorded at -50 mV and in the presence of TTX (0.5 μM), bicuculline (10 μM), picrotoxin (100 μM), strychnine (1 μM) and kynurenic acid (2 mM).
(C) Summary histogram of the desensitization and the amplitude of GABA- and adenosine-induced currents. Desensitization is expressed as the ratio between the amplitude of the current at the end of the agonist application ($I_{(t)}$) and the peak maximal current (I_{max}). The desensitization of $GABA_B$-mediated currents was similar in control and neurons transfected with KCTD8 or KCTD16, but was significantly increased in neurons transfected with KCTD12 (control: 96.2 ± 1.1%, n=19; KCTD8: 95.5 ± 0.9%, n=15; KCTD12: 64.6 ± 2.7%, n=15; KCTD16: 93.7 ± 2.1%, n=10; $^{***}p<0.001$). (KCTD12 compared with control, KCTD16 and KCTD8, ANOVA/Scheffe post-hoc test). In all conditions, the maximal amplitude of $GABA_B$-mediated K^+ currents remained constant (control: 84.3 ± 6.8 pA, n=19; KCTD8: 71.2 ± 10.5 pA, n=15; KCTD12: 81.1 ± 18.1 pA, n=15; KCTD16: 75 ± 19 pA, n=10; p>0.05). As a control, K^+ currents evoked by 100 μM adenosine had similar kinetics (control: 98.9 ± 1.1%, n=4; KCTD8: 97.6 ± 0.6%, n=3; KCTD12: 97.9 ± 0.5%, n=5; KCTD16: 96.7 ± 1.1%, n=7; p>0.05) and amplitudes (control: 33.4 ± 10.2 pA, n=4; KCTD8: 31 ± 2.8 pA, n=3; KCTD12: 29.8 ± 11.3 pA, n=5; KCTD16: 47.8 ± 18.7 pA, n=7; p>0.05) in all conditions. Values are mean ± SEM.

The C-terminus of KCTD12 is critical for the desensitization of $GABA_B$-mediated currents

In addition to the T1-domain, the KCTD proteins contain another highly conserved region in the C-terminus (S Figure 3-2A). To isolate the KCTD12 peptide regions responsible for promoting the desensitization of $GABA_B$-mediated K^+ currents, we transfected hippocampal organotypic slice cultures with two KCTD12 truncation mutants and recorded GABA evoked responses. In neurons transfected with the C-terminus of KCTD 12 ($KCTD12_{v125-\ *328}$, S Figure 3-2A), $GABA_B$-mediated K^+ current kinetic was comparable to non-transfected cells showing that the protein needs to bind to the $GABA_B$ receptor through the T1 domain to induce desensitization (Figure 3-6A, F and G). Similarly, the N-terminus of KCTD 12 ($KCTD_{M1-L125}$, S Figure 3-2A) did not change the $GABA_B$ response kinetic (Figure 3-6B, F and G). Taken together, results of the KCTD12 truncation mutants suggest that the full-length KCTD12 protein is required to alter $GABA_B$ receptor functions.

Consistent with the absence of interaction between $GABA_B$ receptors and KCTD10, we did not observe $GABA_B$ receptor-mediated desensitization with chi10x12 consisting of the N-terminus of KCTD10 and the C-terminus of KCTD12 (Figure 3-6F). But also when the N-terminus of KCTD12 was fused to the C-terminus of KCTD10 (chi12x10), $GABA_B$ response did not desensitize (Figure 3-6F). This supports that binding of KCTD12 to the $GABA_B$

receptor is not sufficient for the observed desensitizing effect, but also suggests that the C-terminus of KCTD12 is necessary to induce desensitization.

Regarding the results obtained by functional assays using truncated KCTD12 mutants, or the chimeric KCTD proteins, chi12x10 and chi10x12, it needs to be considered that they all have their inherent caveats, namely non-interaction due to the truncations and the non-interacting nature of KCTD10. To definitively exclude these pitfalls, we generated additional chimeras: chi12x16 and chi16x12 (S Figure 3-2). As KCTD16 is a $GABA_B$ interacting KCTD but does not induce desensitization of $GABA_B$–mediated of K^+ currents, this makes it a good candidate for generating chimeras with KCTD12. First, we confirmed the interaction of the chi12x16 and chi16x12 with wild-type KCTD12 and KCTD16 (Figure 3-6C) by co-immunoprecipitations from transfected HEK293 cells. Following confirmation of protein interaction, we subsequently recorded $GABA_B$-mediated responses in transfected CA1 pyramidal neurons in hippocampal organotypic slice cultures. Similar to chimera chi12x10, also the chimera chi12x16 where the N-terminus of KCTD12 was fused to the C-terminus of KCTD16, did not induce desensitization of the $GABA_B$–mediated response (Figure 3-6E and F). Strikingly, the chimera containing the N-terminus of KCTD16 and the C-terminus of KCTD12 (chi16x12) induced desensitization of $GABA_B$-mediated currents similar to the full-length KCTD12 (Figure 3-6D and F). We did not observe an effect on the amplitudes of the K^+ currents in any neurons transfected with the different chimeras (Figure 3-6G).

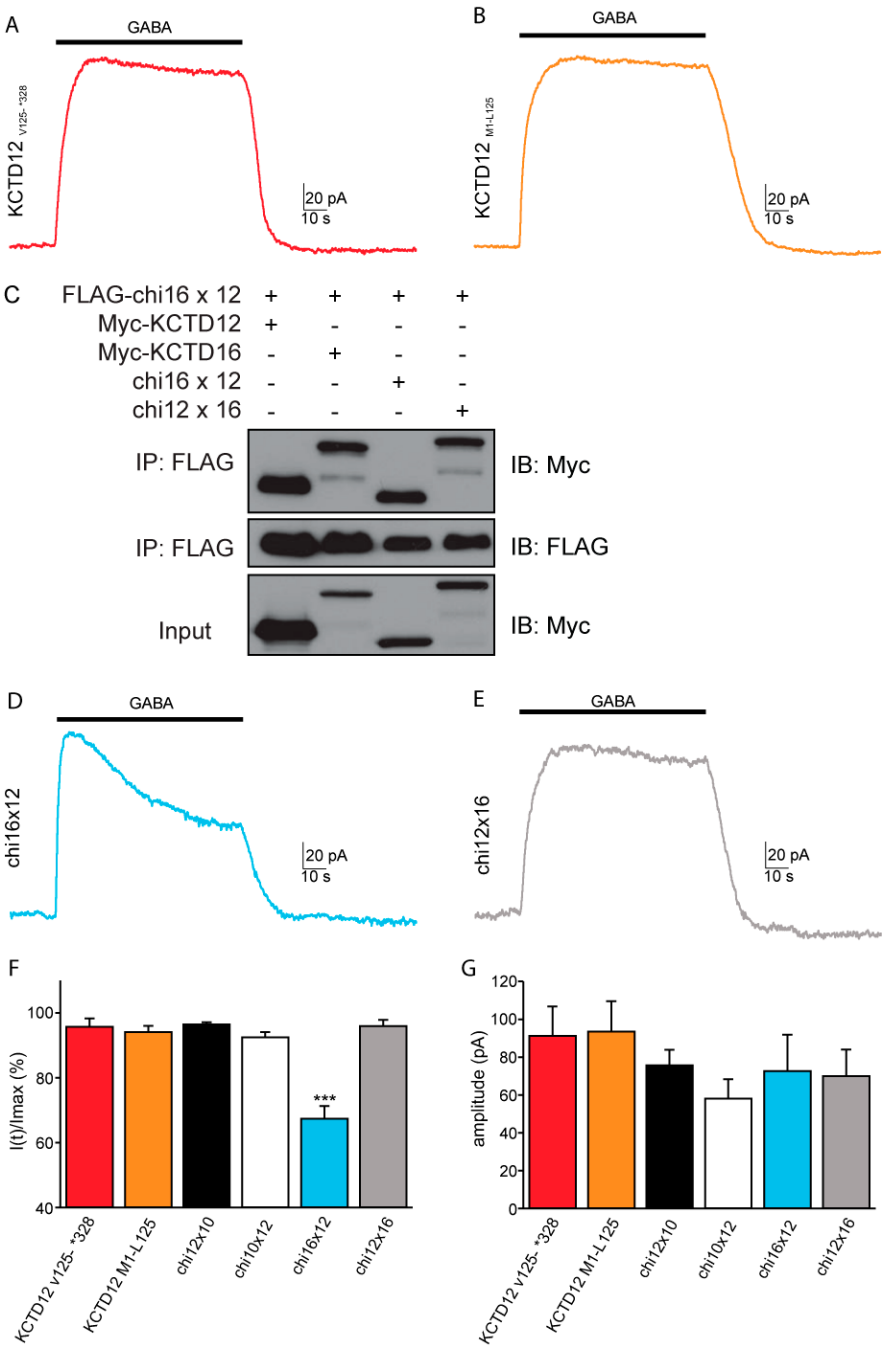


Figure 3-6: The C-terminal domain of KCTD12 is involved in the desensitization of $GABA_B$ response in CA1 hippocampal neurons

$GABA_B$-mediated K^+ currents recorded in CA1 neurons transfected with **(A)** $KCTD12_{v125-\ *328}$ (C-terminus) or **(B)** $KCTD12_{M1-L125}$ (N-terminus). In both conditions, truncated KCTD12 did not increase the desensitization of GABA-induced K^+ currents (A, B and F).

(C) Homo- and heterodimerization of chi16x12 chimeric protein, and wild-type KCTD12 and KCTD16 protein were confirmed by co-immunoprecipitation.

(D-E) Functional analysis of KCTD-chimeric proteins combining the N-terminus of KCTD16 with the C-terminus of KCTD12 (chi16x12) **(D)** and the reverse construct (chi12x16) **(E)**. In CA1 neurons, the chimera, chi16x12, increased the desensitization of $GABA_B$-mediated currents whereas the chimera, chi12x16, had no effect.

(F) Summary histogram showing that cells transfected with the chimera, chi16x12, had a significant increase in $GABA_B$-mediated current desensitization compared with cells transfected with $KCTD12_{v125-}$

$_{*328,}$ $KCTD12_{M1\text{-}L125}$ and chimeras chi12x10, chi10x12, chi16x12, chi12x16, ($KCTD12_{v125\text{-}\ *328}$: 94.2 ± 1.8%, n=6; $KCTD12_{M1\text{-}L125}$: 95.8 ± 2.5%, n=6; chi12x10: 96.6 ± 0.6%, n=16; chi10x12: 93.4 ± 1.8%, n=6; chi16x12: 67.5 ± 3.8%, n=7; chi12x16: 96 ± 1.8%, n=10;, $^{***}p<0.001$, chi16x12 compared with all other conditions, ANOVA/Scheffe post-hoc test).
(G) $GABA_B$-mediated K^+ currents were of similar amplitudes in all conditions ($KCTD12_{v125\text{-}\ *328}$: 91.4 ± 14.9 pA, n=6; $KCTD12_{M1\text{-}L125}$: 93.7 ± 15.4 pA, n=6; chi12x10: 75.8 ± 7.5, n=16; chi10x12: 58.3 ± 9.6, n=6; chi16x12: 72.8 ± 18.5 pA, n=7; chi12x16: 70.1 ± 13.5 pA, n=10; $p>0.05$, chi16x12 compared with all other conditions, ANOVA/Scheffe post-hoc test). Values are mean ± SEM.

To exclude the potential effects of endogenous KCTD proteins in neurons, we performed the same experiments in a CHO cell line stably expressing $GABA_{B1,2}$ receptors and transiently co-transfected Kir3-type K^+ channels (Kir3.1/Kir3.2) with full-length KCTD12 and the chimeric proteins, respectively. In CHO cells transfected with KCTD12, K^+ currents induced by pressure application of 100 μM GABA showed a prominent desensitization compared with currents recorded in control cells. Similar to observations in neurons, the increase in desensitization of the $GABA_B$-mediated response was mimicked when chi16x12, but not when chi12x16 was expressed in the CHO cells (Figure 3-7A and B). In summary, the electrophysiological recordings indicate that KCTD12 mediates desensitization of $GABA_B$–mediated K^+ currents through the C-terminus of the protein.

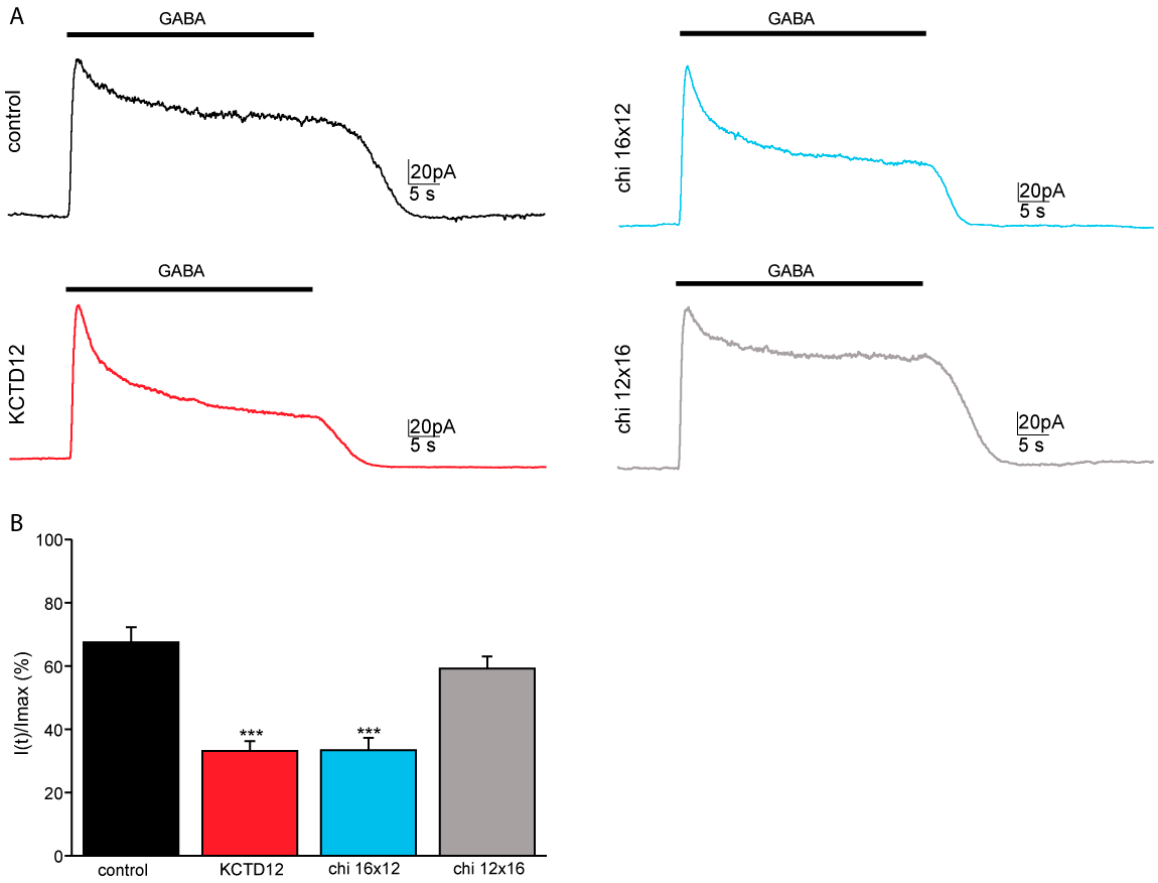


Figure 3-7: When expressed in CHO cells, KCTD12 and the chimera, chi16x12, increase desensitization of $GABA_B$-mediated Kir3-type K^+ currents
(A) Representative traces of K^+ currents induced by pressure application of the agonist GABA (100 µM, 40 s) in CHO cells stably expressing $GABA_{B1,2}$ and transiently transfected with kir3.1/kir3.2 alone (control), co-transfected with KCTD12 or the chi16x12 chimera. Full-length KCTD12 and the chimera, chi16x12, increased the desensitization of $GABA_B$-mediated K^+ current in comparison to control. In contrast, the chimera chi12x16 had no effect on the kinetic of the $GABA_B$-mediated response. Currents were recorded at a holding potential of -50 mV (B) Summary histogram of the desensitization of K^+ currents recorded in CHO cells in control condition, or transiently transfected with KCTD12, chi16x12 or chi12x16. The desensitization of $GABA_B$-mediated K^+ currents was significantly increased in cells expressing KCTD12 and chi16x12 but not chi12x16 (control: 67.5 ± 4.8%, n=9; KCTD12: 33.2 ± 3%, n=8; chi16x12: 33.4 ± 3.9%, n=9; chi12x16: 59.2 ± 3.8%, n= 9, ***p<0.001, KCTD12 and chi16x12 compared with control, ANOVA/Scheffe post-hoc test). Values are mean ± SEM.

Discussion

We have characterized protein oligomerization and subcellular localization of KCTD8, 12, and 16. We identified the KCTD inherent structural requirements for the interactions among the KCTDs, as well as for the interaction with the $GABA_B$ receptor. These $GABA_B$ interacting KCTDs are capable of forming both homo- and heterodimers in heterologous and native tissues. Unlike other T1-domain containing proteins, KCTD8, 12, and 16 do not require the T1-domain for interaction among each other; rather the C-terminal conserved domain is

critical in facilitating protein-protein interaction. Furthermore, the same domain is important for inducing $GABA_B$ receptor-mediated desensitization in cells overexpressing KCTD12.

Critical domains required for KCTD-KCTD and KCTD-$GABA_B$ receptor interactions

A previous study had identified four members of the KCTD family as auxiliary subunits of the $GABA_B$ receptor and had shown that these KCTD proteins are part of a large receptor oligomer (Schwenk et al., 2009); however the study did not address the interaction between KCTDs and the critical domain(s) involved. The large KCTD protein family contains a N-terminal region that is homologous to the T1-domain of voltage-gated K^+ (Kv) channels, a domain that is also homologous to the BTB (bric-a-brick, tramtrack, broad complex) fold; a protein-protein interaction module involved in self-association and interaction with non-BTB partners (Stogios et al., 2005). Based on this, KCTD8, 12, and 16 were hypothesized to form homo- and/or heterodimers. Indeed, results from *in vitro* and *in vivo* protein interaction experiments confirmed this hypothesis. Our data moreover shows that the C-terminus, including a domain that is conserved within the $GABA_B$ interacting KCTDs, is critical for homo- and heteromerization of KCTD8, 12, and 16. An unexpected finding, however, was the non-essential role of the T1-domain in homo- and heterodimerization of the $GABA_B$ interacting KCTD proteins. A recent crystal structure study undertaken by Dementieva et al. supported the concept of oligomerization in KCTDs (Dementieva et al., 2009). Furthermore, the authors showed that KCTD5 crystals form a pentamer instead of the anticipated tetramer. The same study, however, determined the assembly of the KCTD5 pentamer to be mediated via the T1-domain surface. Likely causes for the discrepancies are: different protein expression systems and a truncated recombinant protein were used to demonstrate protein-protein interactions. The T1-domain, however, is not trivial in terms of interaction with $GABA_B$ receptors. Rather it is essential for KCTD12 and 16 to specifically interact with the $GABA_{B2}$ subunit. A critical $GABA_{B2}$-interaction domain could not be mapped for KCTD8 using the chimeras, a result that suggests a native full-length protein conformation as a pre-requisite for interaction. Our study thus points to a KCTD-$GABA_B$ receptor complex whereby the large protein complex is held together by the T1-domain interacting with the $GABA_{B2}$ subunit and the KCTDs associating through their C-termini.

KCTD12 specifically regulate $GABA_B$ receptor-mediated desensitization

Desensitization describes the phenomenon whereby the response of a given receptor declines over time despite the continuous presence of the agonist. It is well known that GPCRs undergo either rapid or slow desensitization (reviewed in Tiao et al., 2001). The

phenomenon was reported as an intrinsic property of $GABA_B$ receptors by Couve et al., whereby the phosphorylation of the $GABA_{B2}$ subunit was claimed to stabilize surface receptors and alleviates receptor desensitization (Couve et al., 2002), however, a definitive mechanism(s) remains elusive. Receptors can either undergo endocytosis or surface redistribution to elicit slow or fast desensitization respectively. $GABA_B$ receptors, however, are relatively stable at the neuronal plasma membrane (Fairfax et al., 2004), which would suggest receptor redistribution as one of the most likely contributor to $GABA_B$ receptor desensitization. A previous report from our laboratory had linked KCTD12 with regulating rapid $GABA_B$ receptor-mediated desensitizing responses (Schwenk et al., 2009). In the present study we aimed to investigate the functional properties of the $GABA_B$ interacting KCTDs within an intact neuronal network, providing a more physiological background. In hippocampal organotypic slice cultures, we analyzed amplitudes and desensitization of $GABA_B$ receptor-mediated K^+ currents. We found $GABA_B$ receptor-mediated desensitization only present when KCTD12 is exogenously expressed, and not upon expression of KCTD8 or KCTD16. In addition, we measured amplitudes and desensitization of $GABA_B$ receptor-mediated K^+ currents in comparison to adenosine evoked K^+ currents. In neither case did we observe an effect on the Kir current activated by adenosine receptor activation, thus confirming specificity of the KCTD12-mediated effect on $GABA_B$ receptors.

We used various KCTD12 truncations and chimeric mutants to map the domain(s) responsible for the desensitizing $GABA_B$ receptor response. Neither the N-terminus, nor the C-terminus of KCTD12 alone was sufficient to induce the desensitizing effect. Most importantly, the chimera, chi16x12, containing the T1-domain of KCTD16 linked to the C-terminus of KCTD12 was able to elicit the $GABA_B$ receptor-mediated desensitizing of K^+ currents, whereas chi12x16 was incapable of eliciting such responses. The critical functional domain(s) thus lies within the C-terminus of the KCTD12 protein. To date, a fast desensitizing $GABA_B$ receptor response is considered to be in the order of minutes (≤5min) (Cruz et al., 2004). This study presented evidence of a novel expedited desensitizing response that can be initiated in the order of seconds. Based on this study, it is postulated that the KCTD12 T1-domain plays a structural role, i.e. to maintain an intact large protein complex similar to T1-domain's role in some Kv channels (Strang et al., 2001, Long et al., 2005, Gulbis et al., 2000) and KCTD5 (Dementieva et al., 2009). In contrast, it is speculated that the C-terminus is responsible for providing functional diversity for the protein complex, likely by acting as high density binding site that interact/traps other membrane and/or surface proteins. It would be intriguing to identify these proteins and elucidate the mechanisms governing $GABA_B$ receptor-mediated desensitization.

Alternative physiological roles for KCTD8 and KCTD16

Our study and previously reported data from Schwenk et al. (Schwenk et al., 2009) have attributed $GABA_B$ receptor-mediated desensitization to KCTD12, however, not all KCTDs that interact with $GABA_B$ receptors have the same function. Overexpressing KCTD8 and KCTD16 in neurons and immortalized cell lines did not induce $GABA_B$ receptor desensitization. However, KCTD8 and 16 were, like KCTD12, shown to increase agonist potency and accelerate the onset of $GABA_B$-mediated Kir responses (Schwenk et al., 2009). KCTD8 and KCTD16, therefore, are likely to regulate other aspects of the $GABA_B$ receptor physiology. Similar conclusions can be drawn from differential localizations of KCTD proteins. Previous localization studies in immortalized cell lines had placed KCTD1 in both the cytosol and nucleus (Ding et al., 2009), KCTD5 (Weger et al., 2007, Bayon et al., 2008) and KCTD11 (Di Marcotullio et al., 2004) in the cytosol, and KCTD10 (Wang et al., 2009) and KCTD13 (He et al., 2001) in the nucleus. In this study, we quantitatively analyzed the KCTD8, 12, and 16 distributions in hippocampal CA1 pyramidal neurons. Like the $GABA_B$ receptors (Vigot et al., 2006), the KCTD8, 12, and 16 proteins are also widely distributed in neuronal compartments, albeit with differential distribution patterns: KCTD8, 12, and 16 displaying enrichment in the spine, and KCTD16 is additionally enriched in the bouton. Unlike some family members, none of the $GABA_B$ receptor-interacting KCTDs was found to be present in the nucleus. The distinct subcellular expression patterns imply that KCTDs can modulate $GABA_B$ receptor physiology based on their differential subcellular distribution. Therefore, the acute regulation-response at the level of the receptor may be a consequence of synergistic actions from all three $GABA_B$ interacting KCTD proteins, KCTD8, 12, and 16.

Materials and Methods

Cell culture and co-immunoprecipitation

HEK293 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% foetal calf serum (FCS) (PIAA), 4500 mg/l of L-glutamine, in a humidified atmosphere of 5 % CO_2 at 37^{o}C. Cells were transfected at 70-80 % confluency using Lipofectamine 2000 (Invitrogen). Typically, for a 6-well transfection reaction, 1 µg of total plasmid DNA, at equal ratios for co-transfection, and 3 µl of Lipofectamine were used (according to the manufacturer's instructions). We tagged KCTDs with either the 3x c-Myc or FLAG epitope at the N-terminus and inserted the cDNAs into the mammalian expression vector pCI (Promega). For co-immunoprecipitations rotein lysates were prepared from cultured cells 48 h following transfection. Following removal of culturing media and washing in PBS, cells were lysed in a modified RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40,

0.5% sodium deoxycholate supplemented with Complete protease inhibitor cocktail (Roche)) on a rotating platform at 4°C for 30 min. The lysate was then cleared by centrifugation at 10,000xg for 20 min at 4°C. Prior to immunoprecipitation, lysate was pre-cleared with protein A sepharose (Amersham) for 1 h at 4°C. Protein A sepharose precoupled with anti-Myc polyclonal antibody was added to the pre-cleared protein lysate and rotated overnight at 4°C. The immune complex was washed 3 times with RIPA lysis buffer, followed by one wash in PBS buffer and then resuspended in an equal volume of 2x Lämmli buffer. Samples were heated to 95°C and centrifuged for 5 min each prior to loading onto 12 % SDS-polyacrylamide gels. For Western blot analysis of immunoprecipitates we used mouse monoclonal anti-FLAG (Sigma) or rabbit polyclonal anti-c-Myc (Convance) and peroxidase-coupled secondary antibodies (Amersham Biosciences). Blots were developed using the enhanced chemiluminescence detection system (Pierce) and exposed to Fuji maximum resolution X-ray films (Fujifilms). For endogenous protein co-immunoprecipitation experiments brain tissue from adult wild-type Balb/c mice was first homogenized mechanically in sucrose buffer (in mM: 320 sucrose, 10 Tris-HCl pH7.4, 0.25 DTT, 1 EDTA; 1% (v/v) SDS) and large cell debris were removed by low speed centrifugation (1000xg for 20 min). Membrane fraction was isolated from the supernatant by ultracentrifugation at 48,000xg for 40 min at 4°C and lysed in modified RIPA lysis buffer for 30 min at 4°C. Lysate was cleared by centrifugation at 15,000xg for 30 min at 4°C. Protein co-immunoprecipitations were performed as described earlier; antibodies used for the co-immunoprecipitation experiments were described previously (Schwenk et al. 2009). Briefly, anti-KCTD8, -12 and -16 specific antibodies raised in rabbit and guinea pig were used for immunoprecipitation and immunoblotting, respectively.

CHO cells and transfection

CHO-K1 cells stably expressing human $GABA_{B1b}$ and rat $GABA_{B2}$ receptor subunits, provided by Novartis Pharma, were maintained in DMEM, 500 μM glutamine, 40 μg/ml L-proline, 0.5 mg/ml G418, 0.25 mg/ml Zeocin and 10% FCS in 5 % CO_2 at 37°C. Concatemers of Kir3.1/3.2 subunits (ref Wischmeyer E et al., 1997) and KCTD12/chimeras were subcloned into pcDNA3.1 and pCI (Promega) vectors respectively. To visualize transfected cells, mutEGFP was included in the transfection mix; cDNAs were cotransfected into 90% confluent cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Hippocampal slice culture and bio-ballistic transfection

Transverse hippocampal organotypic slices were prepared from Wistar rats (400 μm thick) or Balb/c mice (350 μm thick) at postnatal day 3 and 5, respectively (Stoppini 1991) and maintained for 15-20 days in 5 % CO_2 at 37°C. After 7 days *in vitro*, cultures were biolistically co-transfected using the Helio Gene Gun system (Bio-Rad) with GFP-KCTDs and tdimer2-RFP expression vectors (gift from R.Tsien).

All neuronal protein expression was under the control of the neuron-specific human synapsin-1 promoter (gift from K. Svoboda) for 7-8 days. For image acquisition we used a custom-built two-photon laser scanning microscope, based on a BX51WI microscope (Olympus, Japan) and a pulsed Ti:Sapphire laser (Chameleon XR, Coherent, Scotland) tuned to λ = 890 nm, controlled by an open source software package (ScanImage) written in Matlab (Pologruto et al., 2003), and as described by Vigot et al 2006 with minor modifications. Briefly, fluorescence was detected in epifluorescence (LUMPlan W-IR2 60·1.2 NA, Olympus) and transfluorescence mode (achromatic aplanatic condenser, 1.2 NA, Olympus) using 4 photomultiplier tubes (R2896, Hamamatsu, Japan). We used 725DCXR dichroic mirrors and E700SP blocking filters to reflect emitted photons into a secondary beamsplitter, containing a 560DCXR dichroic, 525/50 (green) and 610/75 (red) band-pass filters (AHF Analysentechnik AG, Tübingen, Germany). The slice was placed into a perfusion chamber and superfused continuously (2 ml/min) with ACSF (in mM: 119 NaCl, 2.5 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, gassed with 95% O_2 and 5% CO_2 at room temperature).

For analysis, stacks of images (512·512 pixels) from secondary dendritic branches and thin axons were obtained from transfected CA1 pyramidal neurons (Z-step: 0.5 μm). Maximum intensity projections of green and red stacks were constructed. For the ratio images, we used a hue/saturation/brightness model, where hue was determined by the green/red ratio (using a rainbow color table), and the intensity in the red channel was used to set the brightness. For quantitative analysis, we calculated the green-to-red ratio in a region of interest (dendrite or axon) after subtracting the background fluorescence. To compensate for differences in laser power and expression level, we normalized the ratio in the axon by the average dendritic ratio. Spines were identified by anatomy from tdimer2-RFP images. GFP-KCTD positive spines were defined as having intensity in the green channel at least 3 standard deviations above background.

Electrophysiology

Visualized whole-cell patch-clamp recording was used to investigate $GABA_B$ receptor function. Postsynaptic Kir3-type K^+ currents were recorded 7 days after transfection of hippocampal organotypic slice cultures from the somata of CA1 pyramidal neurons visualized using an infrared-sensitive camera (Till Photonics) and oblique illumination optics (BX51WI; Olympus, Tokyo, Japan). Cells transfected with KCTD proteins and GFP were identified using FITC filter (Olympus). Currents were recorded at 30-32°C in ACSF containing (in mM): 119 NaCl, 2.7 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26,2 $NaHCO_3$, 11 Glucose, pH 7.3, equilibrated with 95%O_2/5%CO_2. The patch pipettes were filled with a solution containing (in mM): 140 K-gluconate, 4 NaCl, 5 HEPES, 2 $MgCl_2$, 1.1 EGTA, 2 Na_2-ATP, 5 phosphocreatine, 0.6 Na_3-GTP, at pH 7.25 (adjusted with KOH). K^+ currents induced by GABA (100 μM) or adenosine (100 μM) were evoked at -50 mV in the presence of TTX (0.5 μM), kynurenic acid (2 mM), picrotoxin (100 μM), bicuculline (10 μM) and strychnine (1 μM). GABA and adenosine were applied for 40 s by a pressure pipette (standard patch pipette; 3 PSI, Picospritzer III, IntracelI). CGP54626A (5μM) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 μM) were applied by superfusion into the recording chamber.

Whole-cell patch clamp recording in CHO cells was performed 24-48 h after transfection in ACSF and with intra-pipette solution described above. $GABA_B$-mediated currents were evoked using pressure application of the agonist GABA (100 μM, 40s) at a holding potential of -50 mV.

Data were acquired with an Axopatch 200B (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz using Digidata 1322A interface (Molecular Devices). Whole-cell currents were analyzed using Clampfit 9.2 (Molecular Devices). CGP54626A received from Novartis Pharma. DPCPX from Tocris Cookson, TTX from Latoxan. All other reagents were from Sigma-Aldrich.

Yeast two-hybrid system

The yeast two-hybrid system used for identifying protein-protein interactions was first described by Field and Song (1989). A modified system based on the LexA DNA binding domain (Bartel et al. 1993) was used in this study with the yeast strain L40 (Invitrogen): *MATa HIS3⊗200 trp1-901 leu2-3,112 ade2 LYS2::(LexAop)$_4$ - HIS3 URA3::(LexAop)$_8$-LacZ GAL4* (Hollenburg et al 1995). For small-scale transformation of yeast, 10 ml of YPAD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 0.1% (w/v) adenine) was inoculated with a colony of L40 yeast. Following overnight incubation at 30°C, the yeast

culture was diluted with 40 ml of YPAD and incubated for a further 2 h to attain mid-log growth phase with an OD_{600nm} of ~1.0. Yeast cells were collected by centrifugation at 1,600xg for 5 min at room temperature and washed twice with 50 ml of TE (10 mM Tris-Cl pH7.5, 0.1 mM EDTA). Yeast cells were made competent by incubating at room temperature for 10 min in solution of 100 mM LiAc in TE. The DNA transformation mix contained 1 µg of plasmid DNA, 10 µg of denatured sheared salmon sperm DNA (Roche, Germany), 100 µl of competent L40 yeast and 700 µl of PEG-solution (100mM LiAc/TE/, 20% (v/v) PEG-4000). The mixture was incubated at 30 ^{o}C for 30 min and dimethyl sulfoxide (DMSO) added to a final concentration of 10% (v/v) prior to heat shocking at 42^{o}C for 7 min. Immediately after heat shock the yeast cells were collected by centrifugation at 10,000xg for 10 sec and cells were washed once with sterile TE before being resuspended in TE buffer. One fifth of the suspension was plated onto yeast drop out media Yc (0.65 % (w/v) yeast nitrogen base, 2 % (w/v) dextrose, 0.5 % (w/v) ammonium sulfate in 1.5% (w/v) agar and supplemented with amino acids as required and incubated at 30^{o}C until colony formation.

β-Galactosidase filter assay. Yeast colonies were picked onto selective yeast media, grown overnight at 30^{o}C and transferred to nitrocellulose filters (Schleicher & Schuell, Germany). Yeast colonies were permeabilised by two rounds of freeze-thawing in liquid nitrogen, before being exposed to Z buffer pH 7.0 (in mM: 60 Na_2HPO_4, 40 NaH_2PO_4, 10 KCl and 1 $MgSO_4$) mixed with 40 mg/ml X-gal, 38 mM β-mercaptoethanol at 30^{o}C. Color intensity was ascertained at 30 min, 1 h and 3 h.

β-Galactosidase agarose overlay assay. To enable uniform and consistent qualitative exposure of yeast cells to X-gal, colonies were picked and resuspended in 100 µl of sterile water. Once resuspended, 5 µl was dotted onto yeast selective media and grown overnight. A solution containing 0.2 mg/ml X-gal, 50 µl/100 ml β-mercaptoethanol, 0.5 M potassium phosphate buffer, 6 % (v/v) dimethylformamide, 0.1 % (v/v) sodium dodecyl-sulphate (SDS) and 5 % (w/v) low melt agarose was layered onto the colonies and the plates were incubated at 30^{o}C until color development.

β-Galactosidase liquid assay. Liquid cultures were assayed for β-galactosidase activity to verify and quantify protein-protein interactions. The β-galactosidase activity was measured using o-nitro-phenyl-β-$_D$-galactopyranosid (ONPG) as a substrate. Overnight cultures of yeast transformants in Yc/-WL were used and the cells from 1 ml of culture were collected by centrifugation at 10,000xg for 10 sec. The cell pellet was resuspended in 700 µl of Z-buffer before being lysed by the addition of 50 µl chloroform and 20 µl of 10 % (w/v) SDS. 160 µl ONPG (0.66 mg/ml) was added and color development was allowed to proceed for 1 h at 30^{o}C. The reaction was quenched by the addition of 400 µl of 1 M Na_2CO_3. After clearing the

reaction by centrifugation at 10,000xg for 10 min, the amount of o-nitrophenol was measured photometrically at 415nm. The following formula was used to calculate β-gal units (Miller, 1972):

Arbitory β−galactosidase unit = 1000 x $\{OD_{415nm}/(t \times V \times OD_{595nm})\}$

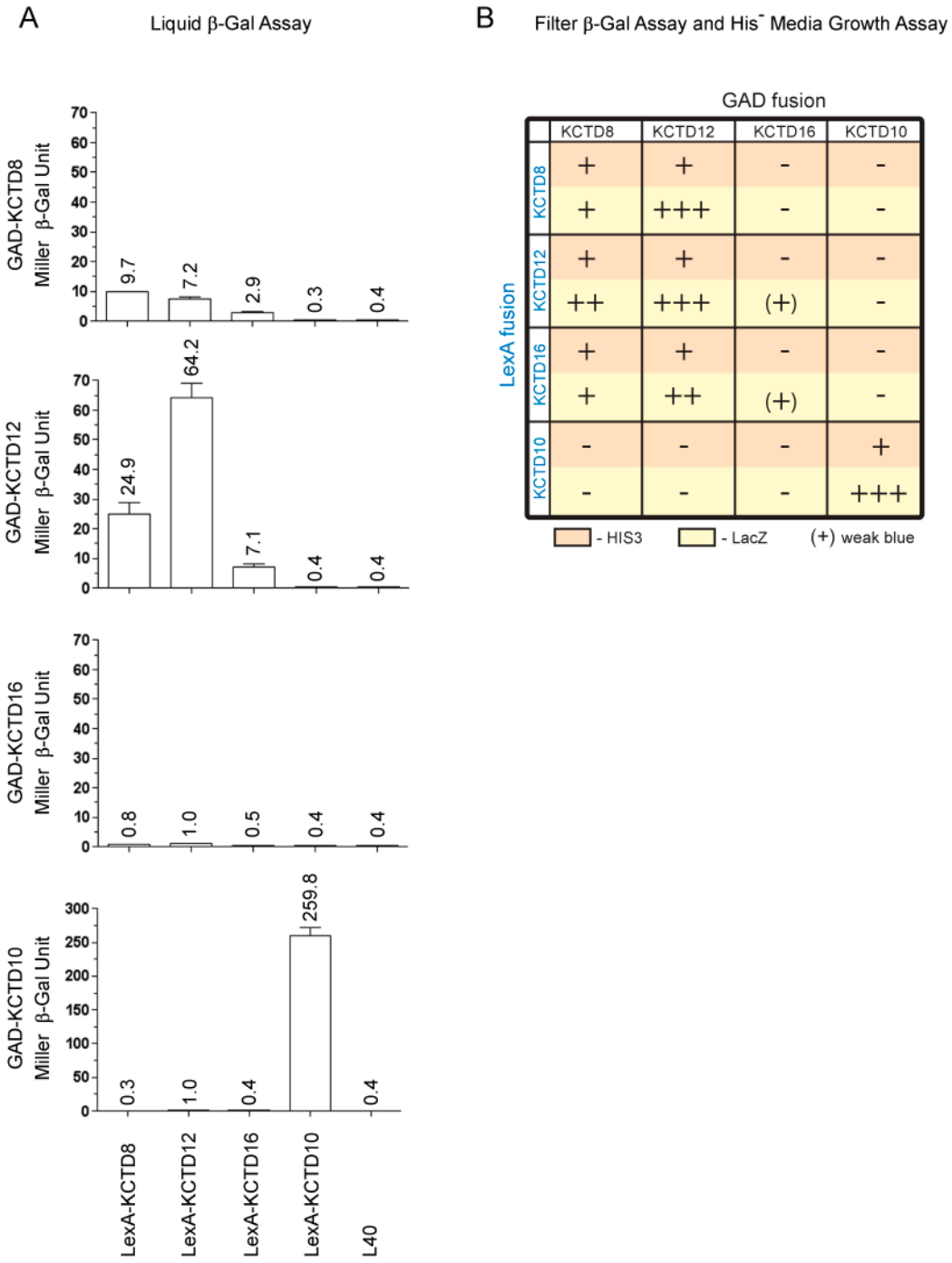
(t = time for yellow color to develop, V = volume of yeast culture used in assay)

Each sample was measured in quadruplicates.

Acknowledgements

We kindly thank Daniela Gerosa-Erni for technical assistance with the organotypic slice cultures.

Supplemental figures



S Figure 3-1: KCTDs were co-expressed in L40 yeast strain to examine protein-protein interaction in a yeast two-hybrid system. Interactions were accessed in both quantitative (A) and qualitative (B) yeast two-hybrid systems. For negative protein interaction controls, KCTD8, 12, and 16 were tested against the $GABA_B$ receptor non-interacting KCTD10. Background reporter activity was verified in L40 alone.

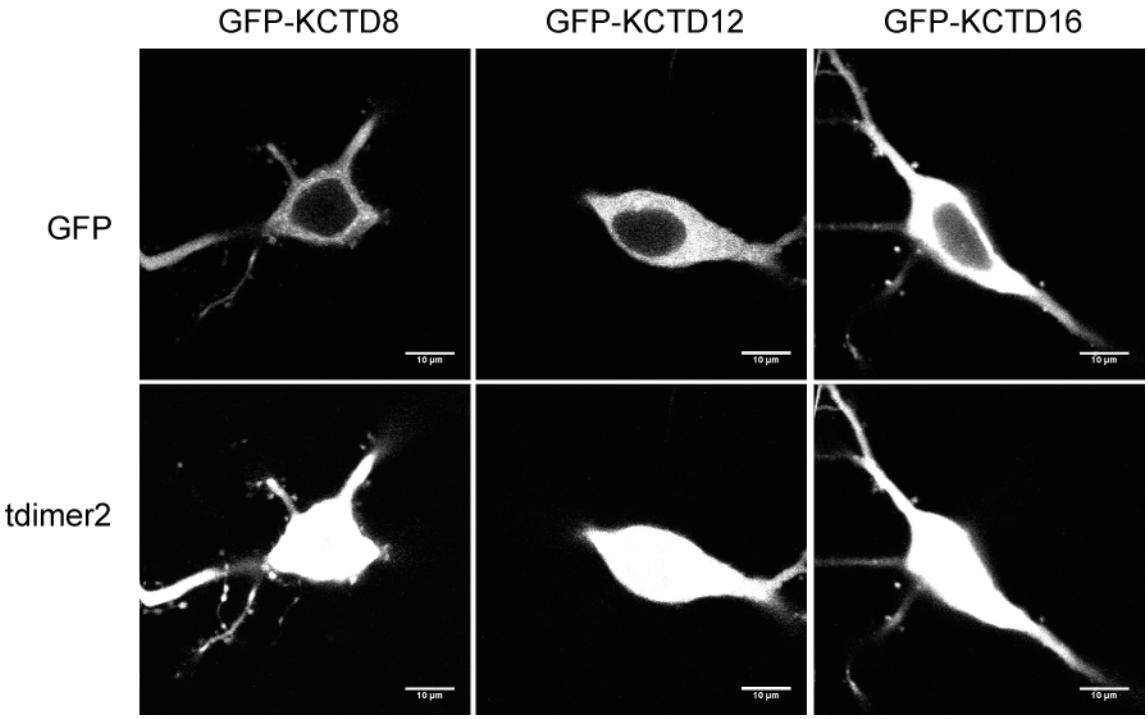
A

```
KCTD8   MALKDTGSGGSTILPISEMVSASSSPGAPLAAAPGPCAPSPFPEVVELNVGGQVYVTKHS 60
KCTD16  MALSGNCS------------------RYYPRDQGAAVPNSFPEVIELNVGGQVYFTRHS 41
KCTD12  MALADSTRG----------LPNGGGGGGGSGSSSSSAEPPLFPDIVELNVGGQVYVTRRC 50
        *** ..                              ... *  **:::*********.*::.

KCTD8   TLLSVPDSTLASMFSPSSPRGGARRRGDLPRDSRARFFIDRDGFLFRYVLDYLRDKQLAL 120
KCTD16  TLISIPHSLLWKMFSPK--RDTAN---DLAKDSKGRFFIDRDGFLFRYILDYLRDRQVVL 96
KCTD12  TVVSVPDSLLWRMFTQQ----QPQ---ELARDSKGRFFLDRDGFLFRYILDYLRDLQLVL 103
        *::*:*.* *  **: .     ..   :*.:**:.***:*********:****** *:.*

KCTD8   PEHFPEKERLLREAEFFQLTDLVKLLSP---------------KVTKQNSLNDECCQSDL 165
KCTD16  PDHFPERGRLKREAEYFQLPDLVKLLAP---------------EDVKQS--PDEFCHSDF 139
KCTD12  PDYFPERSRLQREAEYFELPELVRRLGAPQQPGPGPPPPHSRRGVHKEGSLGDELLPLGY 163
        *::***: ** ****:*:*.:**: *..                  *:.   **    .

KCTD8   EDNVSQGSSDA--LLLRGAAAGAPSGSGAHGVSGVVGGGSAPDKRSGFLTLGYRGSYTTV 223
KCTD16  ED-ASQGSDTR--ICP-------PSSLLPH------------DRKWGFITVGYRGSCTLG 177
KCTD12  AEPEPQEGASAGAPSPTLELASRSPSGGAAGPLLTPSQSLDGSRRSGYITIGYRGSYTIG 223
         :  .* .               ...  .             .:: *::*:***** *

KCTD8   RDNQADAKFRRVARIMVCGRIALAKEVFGDTLNESRDPDRQPEKYTSRFYLKFTYLEQAF 283
KCTD16  REGQADAKFRRVPRILVCGRISLAKEVFGETLNESRDPDRAPERYTSRFYLKFKHLERAF 237
KCTD12  RDAQADAKFRRVARITVCGKTSLAKEVFGDTLNESRDPDRPPERYTSRYYLKFNFLEQAF 283
        *: *********.** ***: :*******:********** **:****:****..**:**

KCTD8   DRLSEAGFHMVACNSSGTAAFVN--QYRDDKIWSSYTEYIFFRPPQKIVSPKQEHEDRKR 341
KCTD16  DMLSECGFHMVACNSSVTASFVN--QYTEDKIWSSYTEYVFYREPSRWSSSHCDCCCKNG 295
KCTD12  DKLSESGFHMVACSSTGTCAFASSTDQSEDKIWTSYTEYVFCRE---------------- 327
        * ***.*******.*: *.:*..  :  :****:*****:* *

KCTD8   DKVTDKGSESGTSCNELSTSSCDSHSEASTPQDNPANTQQAAAHQPNTLTLDRPSRKAPV 401
KCTD16  K--GDKG-ESGTSCNDLSTSSCDSQSEASSPQE--TVICGPVTRQSNIQTLDRPIKKGPV 350
KCTD12  ------------------------------------------------------------


KCTD8   QWMPPPDKRRNSELFQSLISKSRETNLSKKK--VCEKLSVEEEMKKCIQDFKKIHIPDCF 459
KCTD16  QLIQQSEMRRKSDLLRTLTSGSRESNISSKKKAAKEKLSIEEELEKCIQDFLKIKIPDRF 410
KCTD12  ------------------------------------------------------------
```

B

chi8 x 10	Myc_{3x} KCTD8	M1-L142	x KCTD10	L121-*316
chi10 x 8	Myc_{3x} KCTD10	M1-L120	x KCTD 8	V143-*477
chi12 x 10	Myc_{3x} KCTD12	M1-L125	x KCTD10	L121-*316
chi10 x 12	Myc_{3x} KCTD10	M1-L120	x KCTD12	V126-*328
chi16 x 10	Myc_{3x} KCTD16	M1-L118	x KCTD10	L121-*316
chi10 x 16	Myc_{3x} KCTD10	M1-L120	x KCTD16	V119-*428
chi16 x 12	Myc_{3x} KCTD16	M1-L118	x KCTD12	V126-*328
chi12 x 16	Myc_{3x} KCTD12	M1-L125	x KCTD16	V119-*428
FLAG-chi16 x 12	FLAG KCTD16	M1-L118	x KCTD12	V126-*328

S Figure 3-2: Homology between the peptide sequence of KCTD8, 12, and 16 were examined using ClustalW2 multiple alignment tool. The T1-domain (single underline) and the C-terminal conserved domain (double underline) of KCTD8, 12, and 16 are indicated (A). The nomenclature of recombinant mutants used in this study is outlined (B).



S Figure 3-3: Expression of GFP-tagged KCTD8, 12, and 16 in organotypic slice culture. Maximum intensity projections of the soma of a CA1 hippocampal pyramidal neuron expressing GFP-KCTD8, GFP-KCTD12 or GFP-KCTD16 in combination with the freely diffusible tdimer2-RFP are shown. Fluorescence signal from GFP-tagged KCTDs was detected throughout the cytosol, but is absent in the nucleus of the neuron. In contrast, the freely diffusible tdimer2-RFP is ubiquitously expressed. Scale bars, 10 µm.

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Personal contribution

Design of experiments

In vivo heteromerization study

Identification of the $GABA_{B2}$ interaction domain on KCTD8, 12, and 16

Binding study of chi12x16, chi16x12 chimeras

Design of figures

Contribution to the preparation of the manuscript

4 Differential expression pattern of the novel auxiliary subunits of the $GABA_B$ receptor - KCTD8, 12, and 16

(in preparation)

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Abstract

Four members of the potassium (K^+) channel tetramerization domain containing (KCTD) protein family, namely KCTD8, 12, 12b, and 16, were recently identified to be associated with the $GABA_B$ receptor and shown to differentially modulate the kinetics of $GABA_B$ receptor dependent signaling such as activation of G-protein coupled inwardly rectifying potassium (Kir) channels. Here, we describe for the first time a comparative expression pattern of the designated auxiliary $GABA_B$ receptor subunits - KCTD8, 12, 12b, and 16 - in the mouse brain. We performed a systematic expression analysis using in situ hybridization and describe the distribution patterns in context of $GABA_B$ receptor distribution. We demonstrate that the distribution of these $GABA_B$ interacting KCTD isoforms widely overlaps with $GABA_B$ receptor expression and furthermore that the individual KCTD proteins exhibit unique expression patterns in the mouse brain. Comparisons between the adult and postnatal day 5 expression patterns showed that individual neurons express variable subsets of KCTD subunits. In conclusion, this study supports the hypothesis that $GABA_B$ receptors exist as complexes comprising either one or more KCTD and that the $GABA_B$-mediated functional properties of individual neurons are conferred by the specific subset of KCTDs expressed in a cell.

Introduction

Potassium (K^+) channel tetramerization domain containing (KCTD) proteins are an evolutionarily conserved family of cytosolic proteins. Twenty-one isoforms have been identified in humans and 22 in rodents. Their name emerged as the N-termini of KCTD proteins contain a tetramerization domain (T1), which is present in some voltage-gated K^+ (Kv) channels. In Kv channels T1 domains are known to be responsible for subfamily specific co-assembly of the subunits (Xu et al., 1995) and axonal localization (Rivera et al., 2005). Apart from the T1 domain, KCTD proteins share no similarities to the transmembrane ion channel subunits. They are soluble proteins with variable C-termini (Dementieva et al., 2009).

KCTD8, 12, 12b, and 16 were identified as specific interaction partners of the $GABA_B$ receptor ($GABA_BR$) by affinity purification and high-resolution nanoflow liquid-chromatography tandem mass spectrometry (nano-LC MS/MS) analysis and proven to be auxiliary subunits of the $GABA_B$ receptor as they affect the functional properties of the receptor (Schwenk et al., 2009). These four KCTDs share a common structural organization with an N-terminal tetramerization domain, that mediates the interaction with the $GABA_B$

receptor, a central variable linker region, and a conserved C-terminal domain that is involved in homo- and heteromerization of these KCTDs (Tiao et al., 2009).

γ-Aminobutyric acid type B ($GABA_B$) receptors are the G-protein-coupled receptors for GABA, the main inhibitory neurotransmitter in the mammalian central nervous system, and are implicated in a variety of neurological and psychiatric disorders. The functional $GABA_B$ receptor is a heterodimer of the $GABA_{B1}$ and the $GABA_{B2}$ subunit. The $GABA_{B1}$ subunit binds the agonist GABA, whereas $GABA_{B2}$ is necessary for surface trafficking of the receptor and coupling to the G-protein. The activated $GABA_B$ receptor couples to a multitude of signal transduction pathways. $GABA_B$ receptors are involved in the fine tuning of inhibitory synaptic transmission. Presynaptic $GABA_B$ receptors inhibit neurotransmitter release by down-regulating high-voltage activated Ca^{2+} channels, whereas postsynaptic $GABA_B$ receptors decrease neuronal excitability by activating a prominent inwardly rectifying K^+ (Kir) conductance that underlies the late inhibitory postsynaptic potentials. Numerous studies described a large variety of $GABA_B$ mediated effector kinetics observed under physiological conditions. However, the only heterogeneity at the receptor level emerges from the two $GABA_{B1}$ isoforms, $GABA_{B1a}$ and $GABA_{B1b}$. Thus, a major task in recent years aimed to identify the mechanisms underlying different receptor localization and pharmacological properties. Recently, Schwenk et al. demonstrated that the majority of native $GABA_B$ receptors assemble into a high molecular weight complex that constitutively contains either one or more $GABA_B$ interacting KCTD proteins (Schwenk et al., 2009). Binding of KCTDs to the $GABA_B$ receptor could explain the obvious differences in the electrophysiological and pharmacological characteristics of native $GABA_B$ receptors compared with the recombinant receptors. KCTD proteins were shown to determine the pharmacology and kinetics of the $GABA_B$ receptor response. They increase agonist potency and alter the G-protein signaling of the receptor. Interestingly, KCTD proteins induce rapid onset and pronounced desensitization in a subtype-specific manner. For example, all four KCTD proteins accelerate the activation kinetics, while only 12 and 12b markedly increase desensitization of the $GABA_B$ mediated responses. The latest results strongly suggest that the cellular subset of KCTDs assembled with the $GABA_B$ heteromer largely determines the G-protein signaling of the receptor complexes, and thus, generate $GABA_B$ receptors with distinct functional properties. As this is the first described function for this subfamily of KCTDs, we largely emphasize in this study the distribution of the KCTDs in context of the $GABA_B$ receptors. In comparison with the previously described $GABA_B$ receptor localization and function in the analyzed brain structures, we investigated the exact expression pattern of the novel auxiliary $GABA_B$ receptor subunits to further elucidate $GABA_B$ receptor function.

We systematically determined the mRNA expression patterns of KCTD8, 12, 12b, and 16 in the adult mouse brain to identify brain areas that might express only one KCTD or specific subsets of KCTDs, which may consequently display different $GABA_B$ receptor complexes with specific functional properties. Although the biological functions of KCTD proteins remain to be further elucidated, the robust expression of transcripts in fetal tissues in contrast to the low levels in adult, as described for KCTD12 (Resendes et al., 2004), suggest that they play an important role during development. For example, KCTD12 (Pfetin/Ron) is implicated in the maturation of inner ear neurons as indicated by its linkage to a progressive dominant auditory neuropathy (Resendes et al., 2004). The role of $GABA_B$ receptors in brain development, especially in synapse formation and maturation, and the establishing of neuronal circuitries, has emerged during the last years (Lopez-Bendito et al., 2003; Lopez-Bendito et al., 2008; Lujan et al., 2005). Concomitantly, several studies have shown alterations in $GABA_B$ receptor subunit expression and localization during postnatal development (Fritschy et al., 2004; Lopez-Bendito et al., 2002; Lopez-Bendito et al., 2004; Lujan and Shigemoto, 2006). Overall, before postnatal day 10 $GABA_{B1a}$ transcripts are more abundant than $GABA_{B1b}$ transcripts, opposite to what is observed in adult mice (Fritschy et al., 1999; Malitschek et al., 1998). To investigate if KCTDs might potentially be involved in these postnatal changes, we additionally looked at the spatial expression patterns of KCTD8, 12, 12b and 16 transcripts at postnatal day 5.

Materials and Methods

Animals and brain sections

The study was carried out on the brain tissue of adult and postnatal day 5 (P5) BALB/c mice. All animal use procedures were subjected to institutional review and approved by the Veterinary office of the State Basel-Stadt (Switzerland). Mice were anesthetized with isoflurane (Baxter, Deerfield, IL) inhalation prior to decapitation, and the tissues were rapidly dissected, embedded in O.C.T. compound (Sakura Finetek, Zoeterwoude, the Netherlands), and snap frozen in liquid nitrogen. Heads of P5 mice were embedded in whole. Sections of 10 μm thickness were cut with a Microm HM 560 Microtom (Microm, Walldorf, Germany).

Cloning and in situ hybridization

Synthetic digoxigenin-labeled riboprobes (DIG-cRNA) were generated from recombinant pGEM-Teasy (Invitrogen) plasmid containing a cDNA insert of mouse KCTD8 (AY615967, bps 1-216, 1-1088), mouse KCTD12 (NM_177715, bps 2709-4998), mouse KCTD12b (NM_17542, bps 1-298, 1137-1637, 1879-2325, 3233-3708), and mouse KCTD16

(XM_909931, bps2423-4735, 4736-6465). Riboprobes against rat neuron specific enolase (NSE) were generated from recombinant pBluescript plasmid 294 (Schaeren-Wiemers et al., 1997). Transcription was performed from both sides with either T3 or T7 RNA polymerase, generating antisense or sense (control) cRNA probes, respectively. The DIG-labeled cRNA probes were alkaline hydrolyzed to an average length of 200–400 bases. In situ hybridization was performed on cryosections of freshly frozen tissue as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). To classify the expression levels the color reaction for the alkaline phosphatase was stopped at different time points between 24 hours and 4 days.

Image acquisition and processing

Slides were analyzed with a Leica light DMRE transmission microscope (Leica, Wetzlar, Germany) and collected by means of a high-resolution digital camera ColorView IIIu and AnalySIS software (SIS GmbH, Münster). Images were adjusted for brightness and contrast and assembled as plates using Adobe PhotoShop CS2 (v. 9.0.2) and Adobe Illustrator CS2 (v. 12.0.1; Adobe Systems, San Jose, CA). Brain structures were named according to Paxinos (Paxinos G, 2001).

Qualitative and semi-quantitative analysis of expression

The range of observed expression strengths of each KCTD was classified based on six categories: very strong expressing cells (++++), strongly expressing cells (+++), moderately expressing cells (++), weakly expressing cells (+), and cells with no detectable signal (-). A punctuated staining signifying single cell expression pattern was indicated with (o) (Table 4-1). Only KCTDs with strong overall expression and low exposure times were classified as very strong. If gene expression was low and required longer exposure, cells with an exceptionally strong signal were categorized as moderately expressing. Nevertheless, since labeling effectiveness and affinity of the oligonucleotides can vary, a comparative analysis between the different KCTDs is only qualitative. On the other hand, this methodology allows the reliable comparison of expression strength of individual isoforms in different brain regions. The judgments were made relative to the intensity of the NSE hybridization signal (Schaeren-Wiemers et al., 1997). NSE is expressed by all neurons and thus resembles the density of neuronal cells in the different brain areas.

Results

Expression patterns of KCTD genes in adult mouse brain

The spatial expression pattern of KCTD 8, 12 and 16 was analyzed in adult and postnatal day 5 (P5) mouse brain by in situ hybridization (ISH) using digoxigenin-labeled cRNA probes. To compare the differential gene expression patterns in the brain, in situ hybridization analysis was performed on horizontal and coronal brain sections. Riboprobes were preferentially designed in the non-coding and low homology regions of the genes to prevent cross-reactivity. For KCTD8 and 16 two, for KCTD12b four independent probes were used to verify their specificity. For each transcript, the two antisense probes showed the same hybridization pattern, indicating that the antisense probes specifically recognize the corresponding transcripts (data not shown). Sense probes did not show hybridization signals (data not shown). Herein, we summarize the expression patterns of KCTD8, 12, and 16 with an emphasis on particular brain structures. The ISH analysis allows the reliable comparison of expression strength of individual KCTDs between different brain regions (Table 4-1, adult; Table 4-2, P5). However, due to variations in the sensitivity between different probes, a comparative analysis between the different KCTDs can only be qualitative. A summary of the different expression patterns of KCTD8, 12, and 16 is provided in Table 4-1.

Overall, KCTD8 is expressed in only a few brain areas, such as the medial habenula (MH) (Figure 4-1B), the granule cells of the cerebellum and certain brain stem nuclei, whereas KCTD12 and KCTD16 mRNA are widely distributed throughout the whole brain (Figure 4-1C and D, respectively). Transcripts of KCTD12 are primarily observed in the cerebellum, the hippocampal formation (HF) (Fig 1C), and the septum. KCTD16 shows highest expression levels in the cortex (CTX), the thalamus (TH) and the hippocampal formation (Figure 4-1D). The distribution patterns of the KCTDs revealed areas and neurons with different subsets of KCTDs. The pyramidal cells of the hippocampus and dentate gyrus, for example, express high levels of KCTD12 and 16 mRNA, or the medial habenula, which contains KCTD8, 12, and 12b. In contrast, the different cell types of the cerebellum express predominantly the mRNA of one single KCTD subunit. Interestingly, the expression patterns of KCTDs at postnatal day 5 markedly differ to their distribution in the adult, which is particularly evident in the cerebral cortex; an overview is shown in Table 4-2. We performed in situ hybridization analysis also for KCTD12b, and identified that this particular KCTD subunit is only expressed in medial habenula (Figure 4-8E), and is therefore only mentioned in this context. At P5 no KCTD12b mRNA was detected in any of the analyzed tissues.

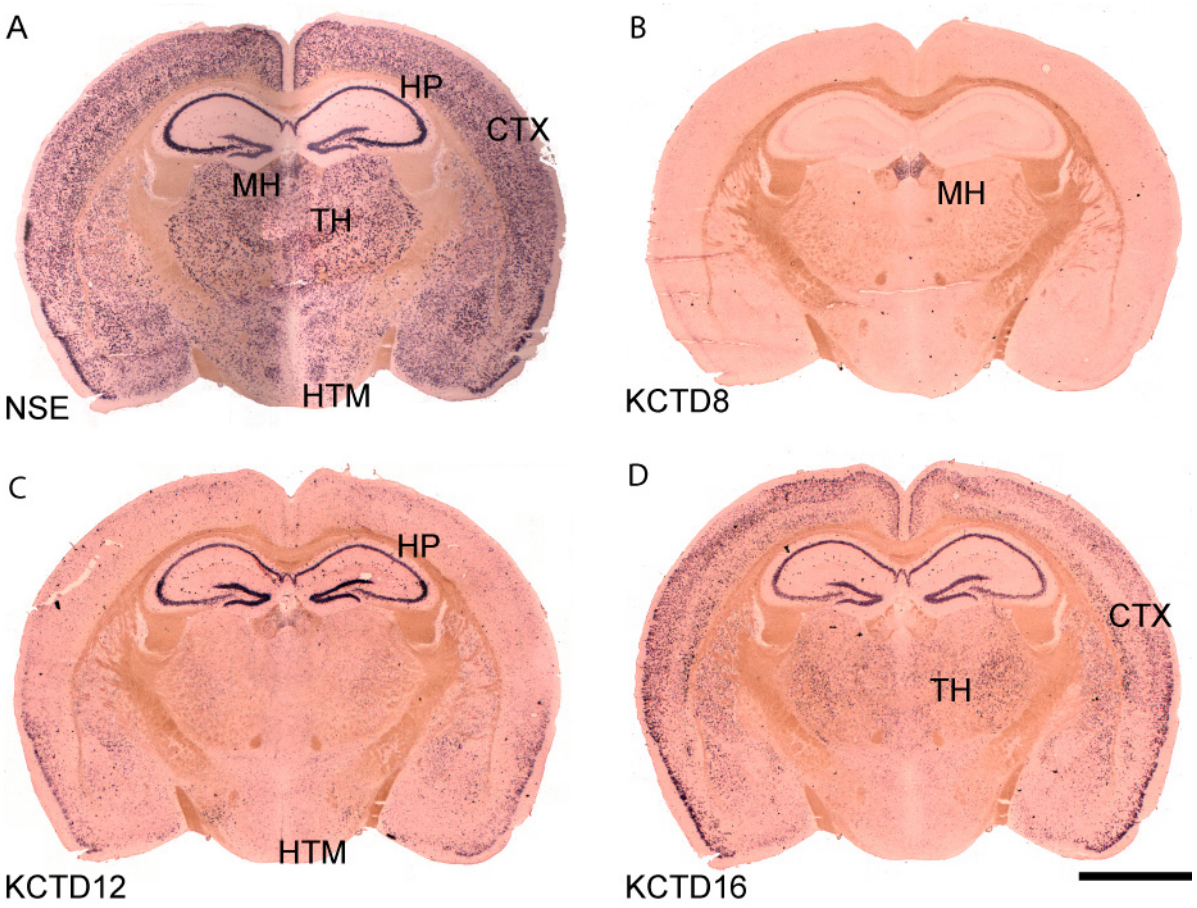

Figure 4-1: Distribution of KCTD mRNA in adult mouse brain. Brightfield images showing the distinct localization of KCTD8, 12, and 16 on coronal sections detected with in situ hybridization (ISH) using digoxigenin-labelled cRNA probes specific for KCTDs compared to the expression pattern of neuron-specific enolase (NSE). CTX, cortex; HP, hippocampus; HTM, hypothalamus; MH, medial habenula; TH, thalamus. Scale bar = 2 mm.

Olfactory system – predominant expression of KCTD12 in ensheating glia

$GABA_B$ receptors show overall a low expression level in the olfactory bulb, but they are strongly expressed in olfactory receptor neurons (ORN) (Panzanelli et al., 2004). Axons of ORNs enter the olfactory bulb via the olfactory nerve layer, and have specifically been shown to contain $GABA_B$ receptors. Generally low hybridization signals were detected for KCTD8 and 16 in the main olfactory bulb; but considerable expression was observed in the glomerular layer (GL; Figure 4-2B, arrows), and in the glutamatergic mitral cells (MC; Figure 4-2D, arrowhead), which are known to form `dendro-dendritic` synapses with GABAergic granule cells providing the `output` stations of the olfactory bulb. The expression pattern of KCTD12, however, exhibits a punctuate pattern in the granular (GCL) and in the mitral cell layer (MCL) (Figure 4-2B), which suggest expression in a subtype of mitral cells (arrow). In contrast to the other two subunits, KCTD12 is highly expressed in the olfactory nerve layer

(Figure 4-2C, open arrows). These non-neuronal cells, presumably ensheating glia cells, strongly express KCTD12 in the adult, as well as at postnatal day 5 (Figure 4-2E). Analysis of P5 animals revealed that KCTD12 and 16 were also expressed in the main olfactory epithelium (MOE) (Figure 4-2F-H). The MOE contains the olfactory nerve neurons in which expression of KCTD12 could be observed, suggesting KCTD12 to be associated with $GABA_B$ receptors in the axons of olfactory receptor neurons (Figure 4-2G, open arrowheads). High levels of KCTD12 and KCTD16 transcripts can also be found in most target regions of the projection fibers of the main olfactory bulb, such as the anterior olfactory nucleus, olfactory tubercle, taenia tecta, piriform and entorhinal cortices (Table 4-1). In the main olfactory bulb, the very high expression of KCTD12 transcripts in olfactory ensheating cells is one of the most striking features (Figure 4-2C and E), which could indicate a role of KCTD12 in glia-neuronal crosstalk, thus influencing guidance of olfactory nerve axons entering the glomeruli of the olfactory bulb.

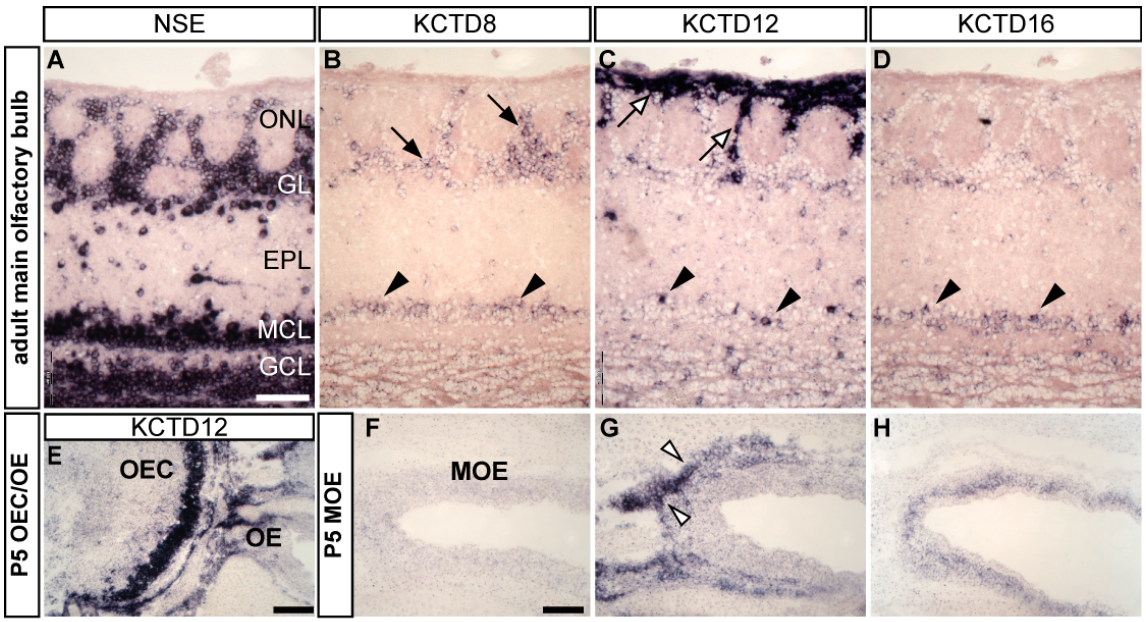

Figure 4-2: KCTD expression in the olfactory system of adult mouse brain. KCTDs exhibit low expression in the main olfactory bulb. The expression pattern of neuron-specific enolase (NSE) is shown on an adjacent section for comparison (A). The non-neuronal olfactory ensheating cells (OEC) show strongest levels of KCTD 12 mRNA (C, open arrows and E). In the main olfactory epithelium (MOE) KCTD12 (G) and 16 (H) are present, KCTD12 in olfactory receptor neurons (G, open arrowheads). EPL, external plexiform layer; GCL, granule cell layer of the olfactory bulb, GL, glomerular layer; MCL, mitral cell layer; ONL, olfactory nerve layer; Scale bars = 200μm.

Table 4-1: Distribution of KCTD8, 12, and 16 in various brain regions. Hybridization signals were analyzed on coronal sections of adult mice. Regions were identified using a mouse brain atlas (Paxinos G, 2001). Intensities of hybridization signals were indicated as follows: ++++, very strong; +++, strong; ++, moderate; +, weak; o, punctate staining signifying single cell expression pattern; -, no detectable signal.

	KCTD8	KCTD12	KCTD16
Olfactory bulb			
Olfactory nerve layer(ensheating glia)	-	++++	-
Glomerular layer	++	+	+
External plexiform layer	-	+	-
Mitral layer	++	++ / o	++
Granular layer	+	+	+
Anterior olfactory n.	-	++	++
Olfactory tubercle	-	+	++
Islands of Calleja	-	-	++
Piriform area, layer II	-	++	+++
Basal forebrain			
Caudate putamen	+	+	+++
Globus pallidus	-	+	-
Nucleus accumbens	+	++	+
Ventral pallidum	-	+	+
Claustrum	-	-	+++
Endopiriform nucleus (ventral part)	-	-	+++
Amygdala			
Basolateral nuclei			
Anterior	-	+	++
Posterior	-	+++	+++
Basomedial nuclei	-	+	+
Central nucleus	+	+++	+
Medial nucleus	+	+	++
Anteroventral	-	-	+
Posterodorsal	+	++	++
Lateral nucleus	-	+	+++
Cortical amygdalar area	-	++	++++
Piriform amygd. Area, layer II	-	++	++++
Bed nucleus stria terminalis	-	+++	+
Septal area			
Lateral septum	-	++++	++
Medial septum	-	+++	-
Hippocampal formation			
Pyramidal layer CA1	+	+++	++++
Pyramidal layer CA2	-	++++	+++
Pyramidal layer CA3	-	+++	+++
Dentate gyrus, granular layer	-	++++	++++
Hilar cells/polymorphic layer	+	+++	+++
Subiculum dorsal/ventral	++	+	++
Entorhinal area, layer II	-	+++	+++
Induseum griseum	-	+++	+++
Taenia tecta	-	++	++
Cortex			
Frontal			
Layer I-III	+	+ / o	+++
Layer V	+	+	-
Layer VI	-	o	++
Layer VIb, isocortex	-	o	++
Parietal			
Layer I-II	+	+ / o	++++
Layer III	-	-	++++
Layer IV	-	-	+++
Layer V	+	+ / o	+
Layer VI	-	o	+++
Layer VIb, isocortex	-	o	+++
Cerebellum			
Molecular layer	-	-	-
Granular layer			
Granule cells	++	-	-
Golgi cells	-	-	+++
Purkinje cells	-	++++	-
Deep cerebellar nuclei	+	+	++

	KCTD8	KCTD12	KCTD16
Thalamus			
Dorsal thalamus			
Anterior group of the dorsal thalamus			
Anterodorsal	-	+	-
Laterodorsal	-	-	+++
Anteroventral	-	-	+++
Medial group			
Mediodorsal n.	+	++	+++
Paraventricular	-	+	-
Ventral group			
Ventral posteromedia	-	++	+++
Ventral posterolateral	-	+	+++
Subparafascicular nucleus	-	+	+++
Posterior group	-	+	+++
Rhomboid n.	-	++	+++
Reunions n.	-	+	++
Reticular thalamic nucleus	-	-	++
Geniculate group			
Medial geniculate nuclei	-	+	++
Lateral geniculate nuclei, dorsal	-	-	+++
Lateral geniculate nuclei, ventral	-	-	+
Medial habenula	++++	+	-
Lateral habenula	+	+	+
Hypothalamus			
Medial preoptic area	-	+++	-
Lateral preoptic area	-	+++	-
Nucleus of the diagonal band	-	++	-
Magnocellular preoptic nucleus	-	+++	+
Dorsomedial hypothalamic	+	+	++
Lateral hypothalamic area	-	++	+
Posterior hypothalamic n.	-	++	+
Mammillary bodies	-	-	++++
Zona incerta	+	+	+++
Midbrain/Pons/Medulla			
Substantia nigra, Pars compacta	-	+	+
Substantia nigra, Pars reticulata	-	+	+
Ventral tegmental area	-	+	+
Interpeduncular nucleus	+	+	++
Mesencephalic reticular nucleus	-	-	-
Olivary pretectal nucleus	-	-	++
Anterior pretectal nucleus	-	+	++
Nucleus of the optic tract	+	+	+
Superior colliculi			
Superficial gray layer	+	+	++
Optical layer	+	+	+++
Periaqueductal grey	-	+	+
Raphe nucleus dorsalis/central	+	+	++
Inferior colliculus	+	+	+++
Parabigeminal nucleus	+	+++	-
N. of the lateral lemniscus	-	+	++
Locus coeruleus	-	+	+
Vestibular nuclei	+	+	+
N. prepositus	+	+	+
Parabrachial n.	+	+	+
External cuneate nucleus	+	++	-
Spinal nucleus of trigeminal	++	++	+
Facial motor nucleus	+	+	+
Dorsal motor n. of the vagus nerve	+	+	+
Hypoglossal nucleus	++	++	+
Dorsal cochlear nucleus	++	+++	+
ventral cochlear nucleus	++	+	+
Pontine reticular nuclei	+	++	+
Superior olivary complex	-	+	+
Inferior olivary complex	-	++	-
N. of the trapezoid body	-	++	-

Basal ganglia – implications for distinct $GABA_B$-KCTD complexes related to Parkinson disease and epilepsy

Different compositions of KCTDs could be identified in the dorsal part of the basal ganglia (caudate putamen and globus pallidus) (for comparison see Table 4-1). For instance the caudate putamen reveals low expression of KCTD8 and KCTD12 mRNA, whereas high expression of KCTD16 mRNA was detected. On the contrary, cells in the globus pallidus only express KCTD12 mRNA, suggesting that KCTD12 could play a specific role in the regulation of pallidal $GABA_B$ receptor activation-dependent regulation of movement and synaptic activity in the basal ganglia known to be a potential therapeutic target for treatment of parkinsonian akinesia (Chen et al., 2008).

In the ventral part of the basal ganglia, KCTD8 is uniquely expressed at low levels in the nucleus accumbens. In the same region, KCTD12 is expressed at moderate and 16 at low levels, whereas the ventral pallidum exhibits low levels of only KCTD12 and KCTD16 mRNA. Intriguing are also the exclusive expression and the very high levels of KCTD16 in the claustrum (CLA) (Figure 4-3D), which is a telencephalic subcortical structure that projects to, and receives projections from a number of cortical areas, including the primary motor, premotor, prefrontal, auditory, and visual cortex (Crick and Koch, 2005). Notably, the claustral complex is involved in propagation of epileptiform activity from the amygdala (Sheerin et al., 2004; Zhang et al., 2001), and the severity of seizures is related to the inhibitory GABAergic activity. Together with findings of Davila et al., which identified a specific GABAergic subpopulation in the claustrum (Davila et al., 2005), the specific expression of KCTD16 might indicate that this subunit may play a role in $GABA_B$ mediated processes that are altered in temporal lobe epilepsies.

Septal area – KCTD12 and 16 are the predominant auxiliary $GABA_BR$ subunits

In the medial septal nucleus (MS) moderate levels of KCTD12 mRNA were observed, whereas KCTD16 mRNA was absent. Neurons in the medial septum are mainly GABAergic and cholinergic and project to the hippocampus. Our data may suggest that the KCTD12 subunit might be involved in modulation of presynaptic $GABA_B$ receptor activation in medial septum, in which activation has been shown to inhibit the generation of frequency rhythmic activity (Henderson and Jones, 2005).

The lateral septal area (LS), a major recipient of hippocampal output, is known to express high levels of $GABA_B$ receptors. Our analysis shows that this particular area has moderate expression levels of KCTD16, whereas high levels of KCTD12 mRNA could be detected

(Figure 4-3G, H). The rostral part (LSr), which contains the highest levels of KCTD12 mRNA, is characterized by the expression of enkephalin and neurotensin and connected mainly with hypothalamic medial zone nuclei, especially the anterior hypothalamic nucleus, indicating that postsynaptically KCTD12 and 16 may be important in $GABA_B$ mediated modulation of hippocampal inputs and presynaptically in the maintenance of body temperature. This point will be revisited again in the section of the hypothalamus. KCTD8 mRNA is not expressed in the septal area (Figure 4-3F). Expression patterns at postnatal day 5 resemble the ones observed in adult mice (Table 4-2). In summary, the analysis showed that KCTD 12 and 16 are the main subunits expressed in the septum.

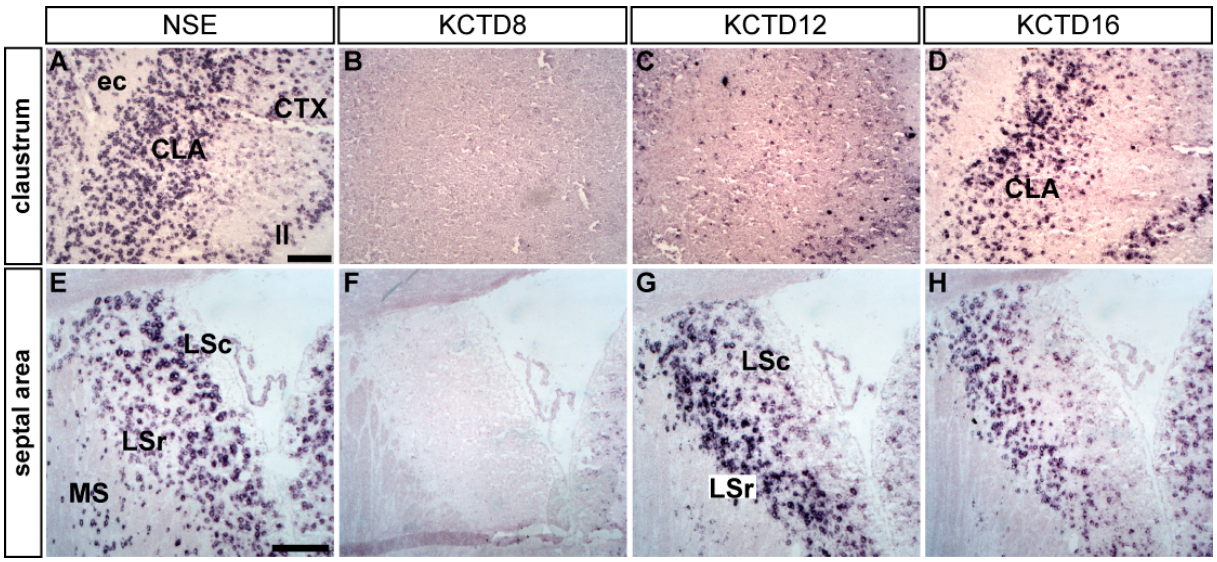

Figure 4-3: KCTD expression in the septal area of adult mouse brain. KCTD12 is expressed at very high (C), KCTD16 at moderate (D) levels in the rostral part of the lateral septum. Exclusive expression of KCTD16 is present in the claustrum (H). Medial septal nucleus, MS; Lateral septum: caudal part, LSc; rostral part, LSr; Caudoputamen, CP. CLA, Claustrum; CTX, Cortex; ec, external capsule. Scale bars = 200μm

Cerebral cortex – differential expression of KCTD12 and 16 at P5 and in the adult

The cortical distribution of $GABA_B$ receptors was extensively studied by in situ hybridization, receptor autoradiography and immunohistochemistry (Bischoff et al., 1999; Margeta-Mitrovic et al., 1999; Princivalle et al., 2000). All studies revealed $GABA_B$ receptor expression throughout the adult cortex in pyramidal cells as well as in interneurons. Binding sites were predominant in the outer cortical layers. In situ hybridization for KCTD8 mRNA revealed that its expression is hardly above background levels, but weak signals in layers II, III and layer V could be detected (Figure 4-4B). In contrast, KCTD12 shows a very distinct expression pattern; on one hand an overall weak signal in layer II and V (Figure 4-4C), on the other hand

strong expression in some sparsely scattered neurons in the layers I and II (Figure 4-4C, inserts), which become more rarely in the deeper layers. According to their appearance, these large spiny neurons are most likely interneurons. KCTD16 transcripts are uniformly distributed throughout all cortical areas. Unlike the other KCTDs, KCTD16 transcripts are found in all cortical layers at high levels, except the layer V, the output layer of the cortex containing the large pyramidal neurons (Figure 4-4D-E). The expression pattern of KCTD12 and 16 transcripts at postnatal day 5 strikingly differ from that in the adult brain. KCTD12 mRNA is concentrated in the inner layers of the cortex, predominantly in layer VI (Figure 4-4H), but also distinct cells in layer I show high expression levels of KCTD12. Conversely, KCTD16 is only expressed strongly in layer III and IV (Figure 4-4I).

$GABA_B$ receptors are expressed early during development and undergo changes in localization during pre- and postnatal development (Lopez-Bendito et al., 2002; Princivalle et al., 2000). The $GABA_B$ receptor antagonist CGP52432 was shown to alter the tangential migration of cortical neurons, which is one of multiple indications for an important role of $GABA_B$ receptors in the migration of cortical interneurons (Lopez-Bendito et al., 2003). What exactly accounts for the coordinated assembly of GABAergic interneurons and projection neurons is still not known. The remarkable expression patterns of KCTD12 and 16 in the cortex at P5 is tempting to speculate that these KCTDs are part of the mechanisms that underlay the last steps of migration and the establishment of synapses.

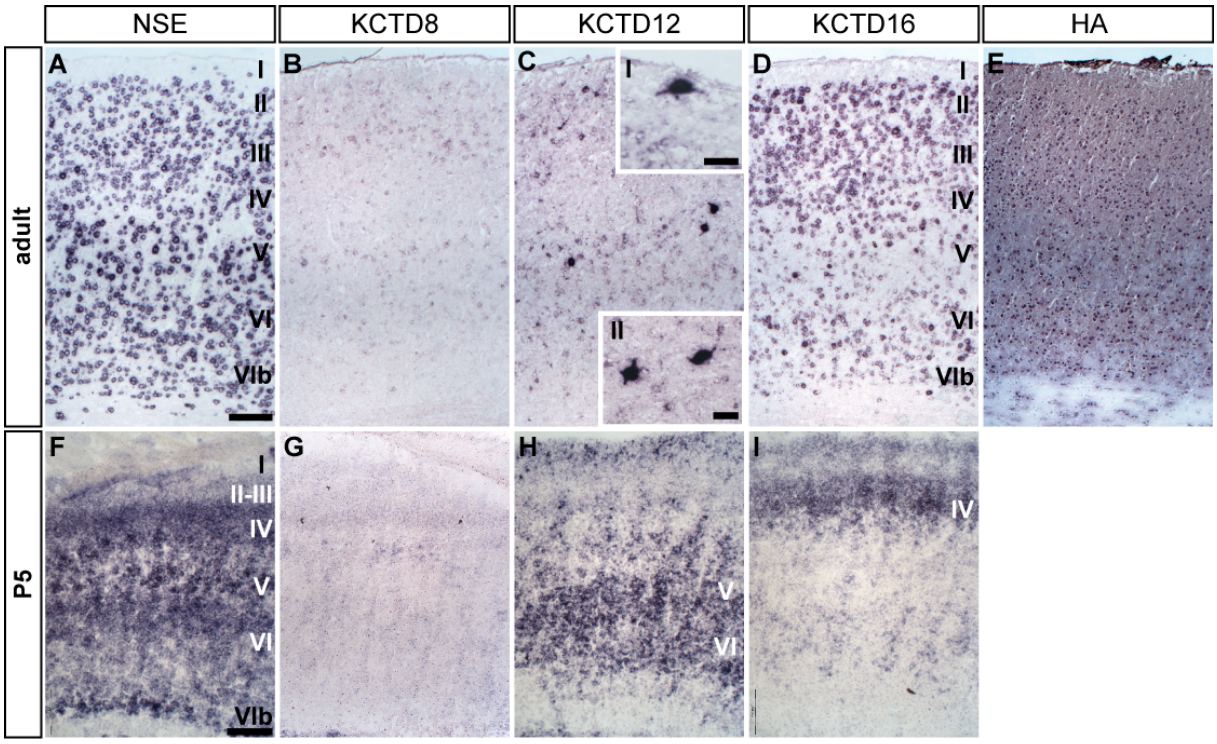

Figure 4-4: KCTD expression pattern within the different layers of the primary sensory area of the cortex. In the adult cortex KCTD12 is highly enriched in scattered interneuron-shaped cells (C), whereas KCTD16 is abundant at high levels in all cortical layers, except layer V (D). Haemalaun (HA) staining is shown on an adjacent section for comparison (E). (P5) KCTD12 mRNA is concentrated the inner (H) and KCTD16 in the outer cortical layers (I). Scale bar = 200µm, 50µm (insert).

Table 4-2: Distribution of KCTD8, 12, and 16 at postnatal day 5 in mouse brain and peripheral tissues. Intensities of hybridization signals were indicated as follows: ++++, very strong; +++, strong; ++, moderate; +, weak; o, punctate staining signifying single cell expression pattern; -, no detectable signal.

Table 2. Summary of the expression of KCTD8, 12, and 16 at postnatal day 5.

	KCTD8	KCTD12	KCTD16
Olfactory bulb	-	++	-
Olfactory nerve layer (ensheating glia)	-	++++	-
Anterior olfactory n.	-	++	+++
granule cell layer	-	++	-
Basal forebrain			
Caudate putamen	++	+++	+++
Globus pallidus	-	+++	-
Claustrum	-	-	+++
Amygdala			
Lateral	++	++/ o	+++
Basolateral	-	+	+++
Central	-	++	-
Septal area	+	+++	++
Hippocampal formation			
Pyramidal layer CA1	+	+ / o	+++
Pyramidal layer CA2	-	+++	+++
Pyramidal layer CA3	-	+++	+
Dentate gyrus, granular layer	-	+++	+
Hilar cells/polymorphic layer	-	+	+
Subiculum dorsal/ventral	++	++	+
Cortex			
I	-	+++	+++
II-III	-	-	+
IV	-	+	+++
V	-	+	-
VI	-	+++	+
Vib	-	-	-
Cerebellum			
Purkinje cell layer	-	+++	+
Deep cerebellar nuclei	-	+	+++
external granule cell layer	+++	+	-
internal granule cell layer	-	+++	+

	KCTD8	KCTD12	KCTD16
Thalamus			
Anterior group of the dorsal thalamus			
Anterodorsal	-	+	-
Laterodorsal	-	-	+++
Anteroventral	-	-	+++
Medial group			
Mediodorsal n.	+	+	+++
Ventral group			
Ventral posteromedial	-	+	+++
Ventral posterolateral	-	+	+++
Subparafascicular nucleus	-	+	+++
Posterior group	-	+	+++
Geniculate group			
Medial geniculate nuclei	-	+++	++
Lateral geniculate nuclei	-	++++	++
Hypothalamus			
Mammillary bodies	-	-	++++
Midbrain/Pons/Medulla			
Superior collicullus	+	++	+
Inferior colliculus	+	++++	++
Parabigeminal nucleus	-	+++	-
Dorsal cochlear nucleus	++	+++	+
ventral cochlear nucleus	++	+	+
N. of the lateral lemniscus	-	+	++
Superior olivary complex	+++	+	++
Spinal nucleus of trigeminal	++	++	+
Peripheral tissues			
Retina	++++	+++	++
Olfactory epithelium	-	++	+++
Spiral ganglion/ Cochlea	++	++++	+
Trigeminal ganglion	++	+/ o	++++
Inferior glossopharyngal ganglion	-	++++	++
Whisker	-	+++	-
Dental pulp	-	+++	-
Bone marrow	-	++++	-

Amygdala - Nuclei expressing different subsets of KCTDs

The primary functional role of the amygdala is thought to be the formation and storage of memories associated with emotional events. It is one of the brain structures that exemplarily exhibit very distinct KCTD expression patterns from low to very high levels in the different nuclei of the amygdala. In the lateral amygdala nucleus (LA) and the posterior basolateral nucleus (BLA) KCTD16 transcripts were highly abundant (Figure 4-5D, H), whereas KCTD12 transcripts were detected primarily in the central amygdala nuclei (CEA), in a subpopulation of cells in the BLA (Figure 4-5C, G) and in the posterodorsal part of the medial nuclei (Table 4-1). The lateral amygdala receives sensory input from thalamic and cortical processing stations, which have been implicated in the associative conditioning of fear responses to sensory stimuli (LeDoux, 2007). The amygdala is characterized by a strong inhibitory tone

that is assumed to be necessary for the appropriate response to sensory signals. $GABA_B$ receptors were recently shown to be involved in the inhibition of glutamatergic inputs to the lateral amygdala (Pan et al., 2009) and also in amygdala LTP (Shaban et al., 2006). Our data might suggest that KCTD16 could play a role in modulating $GABA_B$ receptor function in the lateral amygdala nucleus, i.e. by increasing the agonist affinity of the receptor (Schwenk et al., 2009). On the other hand, the sparsely scattered cells expressing KCD12 in the BLA, which most likely resemble local interneurons, may suggest a role of KCTD12 in the differential effects of $GABA_B$ autoreceptor activation observed at GABAergic synapses in the BLA (Silberman et al., 2009).

Principal neurons in the LA and BLA connect to the central nucleus, which in turn projects to the brain stem autonomic nuclei and/or via the bed nucleus of the stria terminalis, mediating cardiovascular responses to sensory stimuli. $GABA_B$ receptors are critically involved in the strong inhibitory tone observed in the CEA (Ciriello and Roder, 1999). The central amygdala nucleus is one of the few amygdala nuclei which, in addition to KCTD12 and 16, also express low levels of KCTD8 mRNA (Figure 4-5B, arrow). CEA and the bed nucleus of the stria terminalis exhibited high levels of KCTD12 mRNA, which may add to the strong $GABA_B$ mediated functions in these nuclei (Table 4-1).

The cortical amygdala areas express KCTD12 at high and KCTD16 at very high levels (Table 4-1). As the cortical amygdala nuclei receive input from the olfactory bulb and olfactory cortex and are mainly involved in the sense of smell and pheromone-processing, the strong expression of KCTD12 and 16 supports a potential role in the processing of sensory information.

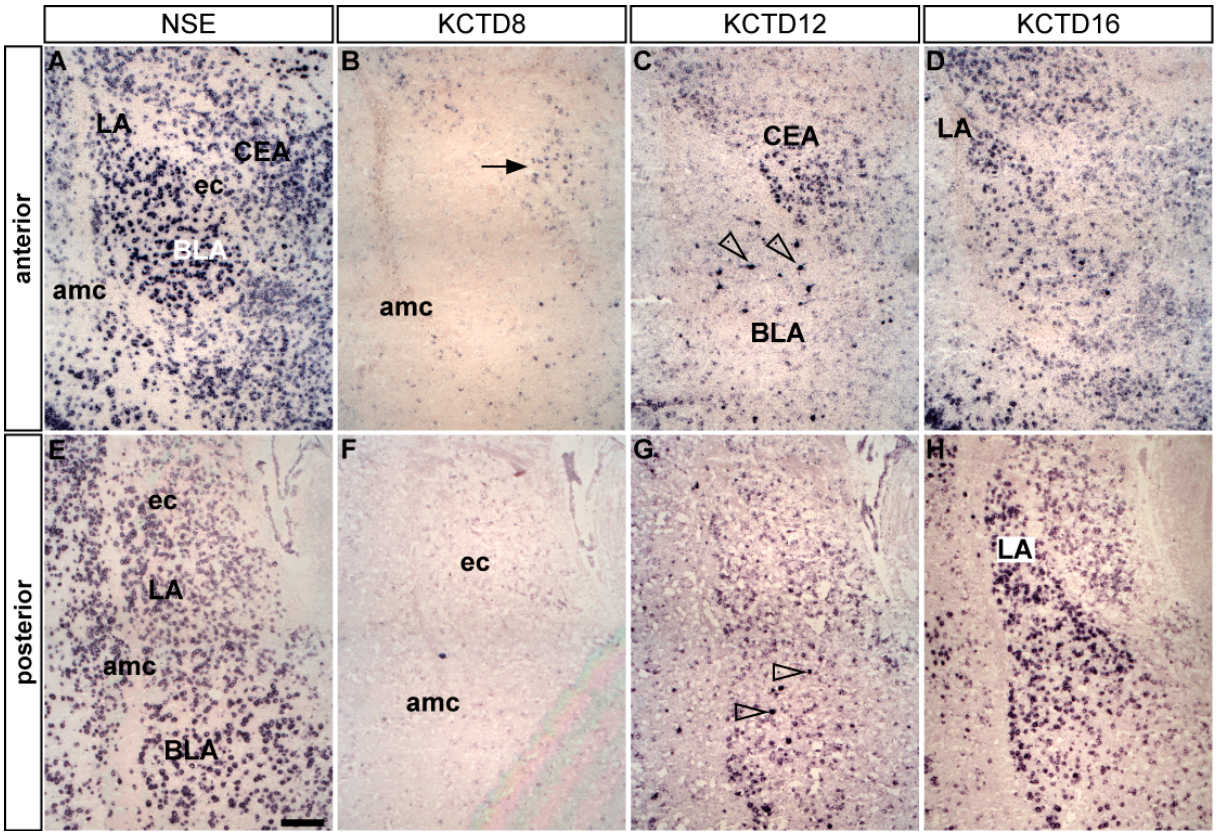

Figure 4-5: KCTD gene expression pattern in anterior and posterior nuclei of the adult mouse amygdala. The central amygdala (CEA) shows strong expression of KCTD8 (B, arrow) and very high levels of KCTD12 (C), whereas KCTD 16 is the predominant isoform in the lateral amygdala (LA) (D and H). In the basolateral amygdala (BLA) a subgroup of cells strongly express KCTD12 mRNA (C and G, open arrowheads). Amygdalar capsule; ec, external capsule. Scale bar = 200 µm

Hippocampal formation – KCTD12 and 16 as main auxiliary $GABA_BR$ subunits

$GABA_B$ receptors are involved in many important functions, such as in long-term potentiation (Davies et al., 1991), in the control of frequency and synchronization of theta rhythm (Davies et al., 1990; Mott and Lewis, 1991), and in the mediation of the late phase of the inhibitory postsynaptic potentials (Dutar and Nicoll, 1988; Karlsson and Olpe, 1989; Olpe et al., 1993). Many studies have confirmed an ubiquitous expression of $GABA_B$ receptors in the hippocampus, in adult as well as during postnatal development. Some studies have focused on the distribution of the $GABA_{B1a}$ and $GABA_{B1b}$ subunits (Kulik et al., 2003), and have shown that the $GABA_{B1a}$ subunit mediates heterosynaptic depression at glutamatergic terminals (Guetg et al., 2009). Overall, $GABA_B$ receptors are localized pre- and postsynaptically at both glutamatergic and GABAergic synapses during postnatal development (Lopez-Bendito et al., 2004). Similarly, comparable distribution has been observed in the adult (Kulik et al., 2003). Thus it is expected that the hippocampus is one of the areas with the strongest expression of the $GABA_BR$ auxiliary subunits.

Indeed, our analysis shows that KCTD12 and 16 mRNA are present at very high levels in the pyramidal cell layer of the hippocampus and the granular layer of the dentate gyrus (DG). Strongest KCTD12 hybridization signals were observed in the pyramidal neurons of the CA2 and CA3 region, and the dentate gyrus (Figure 4-6B), whereas strongest KCTD16 expression was observed in the pyramidal neurons of the CA1 region and in the dentate gyrus (Figure 4-6C). In addition to pyramidal cells, there is a population of basket cells of various sizes and shapes that have their cell bodies located in the pyramidal cell layer, some of them showing strong hybridization signals for KCTD12 and 16 (Figure 4-6B, C insets). There are also a variety of non-pyramidal cell types in the stratum oriens, stratum radiatum, and stratum lacunosum-moleculare of the hippocampus known to be immunoreactive for GABA, and most are considered to be local circuit interneurons. Cells in these layers, as well as in the hilus of the dentate gyrus exhibit high expression levels of KCTD12 as well as 16 (Figure 4-6B, C, arrows).

In the subiculum (S) KCTD16 mRNA is present at high levels, whereas KCTD12 mRNA was only detected at very low levels. The neurons of the subiculum, which constitute the main output of the hippocampus, also contain KCTD8 mRNA (Figure 4-6A). The subiculum sustains afferent and efferent connections to the entorhinal cortex. The outer layers (layer II) of the entorhinal area contain high KCTD12 and 16 transcript levels (Table 4-1; Figure 4-3C, D). Layer II contains mainly stellate cells that are the principal source of fibers for the perforant pathway projection to the dentate gyrus and the CA3.

At P5, KCTD8 mRNA is present in the hippocampus at moderate levels, primarily in the subiculum (Figure 4-6D). KCTD12 message is very high in hippocampal CA2 and CA3 region, the dentate gyrus and in non-pyramidal cells in the stratum oriens, stratum radiatum and the stratum lanculosum-moleculare (Figure 4-6E). KCTD16 was mainly present in the pyramidal layer of the CA1 and CA2 region, but only at moderate levels (Figure 4-6F). The KCTD expression pattern at P5 partially overlap with the patterns observed in adult animals, but additionally exhibit noticeable preferences. Presumably the localization of different $GABA_B$-KCTD complexes is important during hippocampal development, and in the formation of neuronal circuits in the hippocampus.

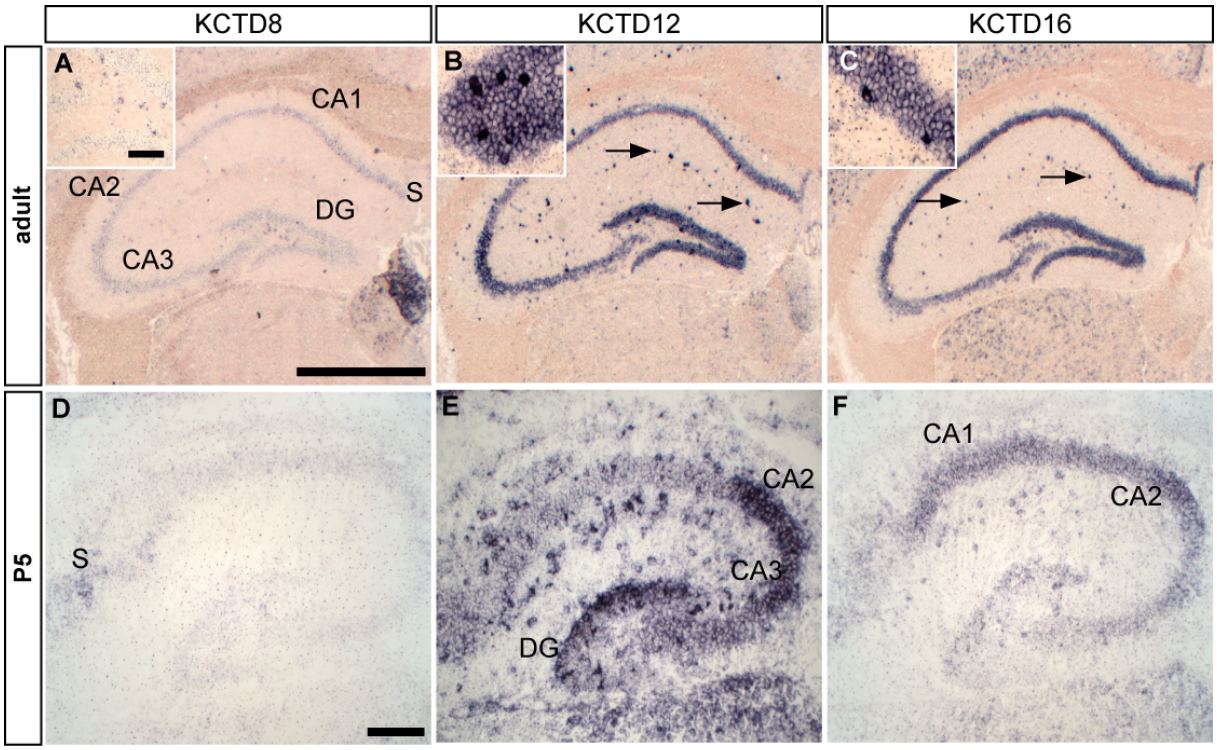

Figure 4-6: KCTD gene expression pattern in the hippocampus of adult and P5 mice. Low levels of KCTD8 are expressed in the subiculum (S) and in hilar cells of the dentate gyrus (DG) (A, insert). KCTD 12 and 16 exhibit strong expression in the pyramidal neurons and interneurons of the hippocampus (B and C, arrows, inserts). (P5) KCTD8 mRNA is present in the subiculum (D). Highest KCTD12 mRNA levels are present in pyramidal cells of CA3 and DG (E), whereas the CA1 field shows highest KCTD16 expression (F). CA1, CA1 field of hippocampus; CA2, CA2 field of hippocampus; CA3, CA3 field of the hippocampus. Scale bar = 1000μm (A-C) ; 50μm (insert A-C); 200μm (D-F).

Thalamus – KCTD16 is the predominant auxiliary $GABA_BR$ subunit in the thalamus

The thalamus is an essential relay point for the processing of somatosensory, visual, and auditory sensory input to the cortex. In general, the thalamus is one of the highest $GABA_BR$ expressing areas in the brain. Porter and Nieves have demonstrated that presynaptic activation of $GABA_B$ receptors modulates thalamocortical excitation of inhibitory and excitatory neurons and provide another mechanism by which cortical inhibition can modulate the processing of sensory information (Porter and Nieves, 2004). Prominent mRNA expression was also observed for the $GABA_B$ interacting KCTDs. The nuclei in the anterior group of the dorsal thalamus express only one KCTD (Figure 4-7B-D). The anterodorsal nuclei contain low levels of KCTD12 mRNA (Figure 4-7C, arrow), whereas the laterodorsal and the anteroventral nuclei contain high levels of KCTD16 mRNA (Figure 4-7D, arrowheads). A high number of KCTD16 expressing cells were also found throughout the thalamic nuclei in the medial and the ventral part. The mediodorsal nucleus of the medial group is one of the few nuclei in the thalamus, where KCTD8, 12, and 16 are expressed, with

KCTD16 showing the highest expression (Figure 4-7B-D). The posterior group, the medial and laterodorsal geniculate nucleus, and the subparafascicular nucleus show moderate to high levels of KCTD16 transcripts (Figure 4-7F-H, Table 4-1). In contrast to the cortex, amygdala and hippocampus, expression of KCTD12 was low and only present in a few nuclei of the thalamus, such as the nuclei of the medial and the nuclei of the ventral group of the dorsal thalamus, and the medial geniculate nucleus (Table 4-1, Figure 4-7C and G). KCTD16 mRNA is abundant in almost all thalamic areas and nuclei at moderate to high levels (Figure 4-7D and H), whereas KCTD8 is absent from most thalamic nuclei (Figure 4-7B and F). From our data, we suggest that most thalamic $GABA_B$ receptors complexes would contain only KCTD16 as auxiliary subunit, supporting a potential function in $GABA_B$ mediated modulation of the processing of sensory inputs.

The overall expression of KCTD transcripts in the thalamus at P5 resembles the expression pattern observed in the adult brain (Table 4-2). In the dorsal thalamus KCTD16 is expressed at high levels, whereas KCTD12 is observed only in the anterodorsal nuclei of the anterior group, in the medial and ventral group and at low levels. What differs from the expression at adulthood is the strong expression of KCTD12 transcripts in the geniculate group, which transfers auditory and visual inputs to the thalamus. This could hint to a function of KCTD12 in the development of sensory pathways. Especially since KCTD12 was detected in cells of both the cochlea and the retina (Figure 4-9B and H).

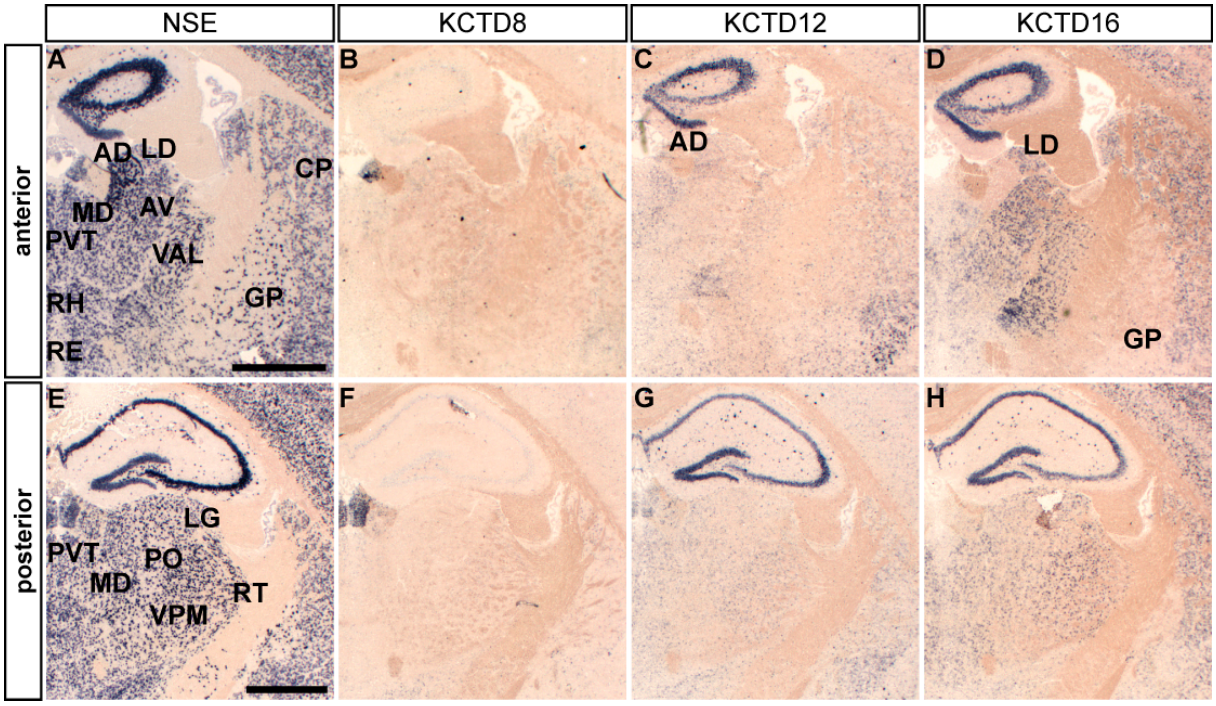

Figure 4-7: KCTD gene expression pattern in the thalamus of the adult mouse. KCTDs exhibit distinct expression patters in the striatum and the anterior and posterior thalamus of adult mouse

brain. The striatum contains strong KCTD12 expression (C), KCTD16 is expressed stronly in the caudatoputamen (CP), but absent from the globus pallidus (GP) (D). KCTD16 shows high expression levels in the anterior thalamic nuclei, excluding the anterodorsal nucleus of the thalamus (D). KCTD16 is abundant at moderat to high levels throughout the posterior thalamus (H). AD, anterodorsal n. of th.; AV, anteroventral n. of th. ; LD, laterodorsal n. of th.; LG, lateral geniculate n.; MD,mediodorsal n. of th.; V AL, mitral cell layer; PVT, paraventricular n. of th.; PO, posterior group; RH, rhomboid n.; RE, reunions n. ; R T, reticular thalamic n.; VPM, ventral posteromedial n. of th.. Scale bars = 1000 µm.

Epithalamus – a brain region with strongest expression of KCTD8 and 12b

The medial habenula (MH) is a cholinergic nucleus projecting through the retroflex fasciculus to the interpeduncular nucleus (habenulointerpeduncular tract). The function of the habenulointerpeduncular connection is poorly understood, although there is evidence from rodents that it modulates complex behaviors such as avoidance, reward and feeding (Sutherland, 1982). However, it is among the brain areas that contain the largest amounts of $GABA_B$ receptors, but in contrast to most other thalamic nuclei it contains the highest levels of KCTD8, as well as KCTD12 mRNA (Figure 4-8B, C). Of particular interest is the exclusive expression of KCTD12b mRNA in this region (Figure 4-8E). The lateral habenula contain low levels of KCTD16 mRNA, whereas the medial habenula does not exhibit any KCTD16 expression (Figure 4-8D).

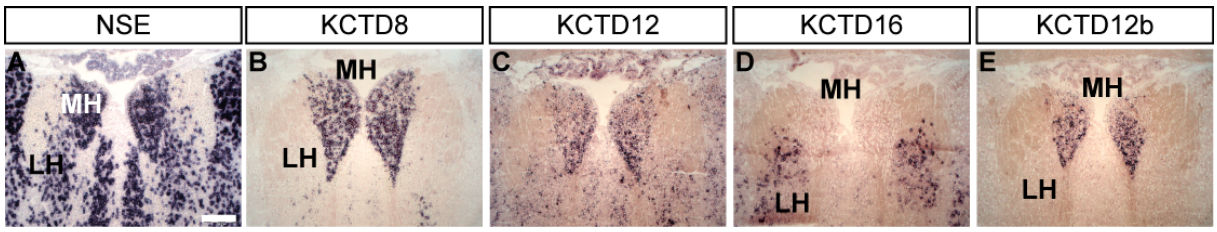

Figure 4-8: KCTD gene expression pattern in the habenula of the adult mouse. KCTD8 (B), 12 (C), and 12b (E) are expressed highly in the medial habenula. KCTD16 (D) mRNA is only present in the lateral habenula. Scale bar = 200 µm.

Hypothalamus – KCTD12 abundant at low levels

In contrast to the thalamus, KCTD12 transcripts are more abundant in the hypothalamus (Figure 4-1C, Table 4-1). KCTD12 mRNA is highly expressed in the anterior part of the hypothalamus, in the medial and the lateral preoptic area, the nucleus of the diagonal band and the magnocellular preoptic nucleus. Moderate levels of KCTD12 were also detected in the lateral hypothalamic area and the posterior hypothalamic nuclei. As mentioned previously, the anterior nuclei receive input from the lateral septum and control the maintenance of body temperature. The specific $GABA_B$ agonist baclofen is well known to

induce hypothermia (Badran et al., 1997). Moreover analysis of the basal body temperature showed that $GABA_{B1}$ knockout mice are hypothermic by approx 1°C when compared to wild-type mice (Schuler et al., 2001). This is likely caused by the loss of $GABA_B$ function in the hypothalamus, where $GABA_B$ receptors are involved in the control of body temperature (Pierau et al., 1997). Other areas of the hypothalamus showed no or very low levels of KCTD12. KCTD8 and 16 were not present in most of the nuclei. High levels of KCTD16 mRNA could only be detected in the zona incerta and very high levels in the mammillary bodies. These observations go in line with the hypothalamic areas that highly express $GABA_B$ receptors. Particularly some of the preoptic nuclei and the mammillary bodies (Margeta-Mitrovic et al., 1999). A strong expression of KCTD16 transcripts in the mammillary bodies is already present at postnatal day 5 (Table 4-2).

Midbrain/brainstem/peripheral tissue – particular expression pattern of KCTDs in the sensory system

$GABA_B$ receptors are expressed in several regions of midbrain and brainstem, as well as in peripheral tissues (Ong and Kerr, 1990). They are expressed in primary sensory neurons, the spinal cord and in several relay nuclei of sensory pathways (Durkin et al., 1999; Margeta-Mitrovic et al., 1999). Generally KCTD12 and KCTD16 transcripts show low expression levels in the midbrain with few exceptions, whereas KCTD8 transcripts exhibit highest expression levels in certain brainstem nuclei. The strong expression of KCTDs in some of these areas suggests a role in modulating $GABA_B$ action in a variety of sensory modalities.

Visual system. Similar to $GABA_B$ receptors (Koulen et al., 1998), KCTD8, 12, and 16 transcripts are present in various neurons of the retina (Figure 4-9A-C). Our analysis detected KCTD12 and 16 mainly in retinal ganglion cells, whereas KCTD8 and 16 are present in cells of the inner nuclear layer that contains amacrine and bipolar cells. In addition, KCTD16 is highly expressed in the target regions of the retina, such as the anterior pretectal nucleus, the superior colliculus and the dorsal lateral geniculate nucleus (Table 4-1). We found moderate levels of KCTD8 and very high levels of KCTD12 mRNA also in the parabigeminal nucleus (PBN), a subcortical visual center projecting to the superior colliculus (SC) and the amygdala (Figure 4-9D-F). The majority of cholinergic input to the superior colliculus originates in the parabigeminal nucleus (Mufson et al., 1986), which is also rich in $GABA_B$ receptors (Margeta-Mitrovic et al., 1999).

Auditory system. Resendes et al. were the first to clone KCTD12 from a human fetal cochlea library and implicated an auditory system related function for KCTD12. We found KCTD12 to be highly expressed in the cochlea (Figure 4-9E), supporting their hypothesis. According

Resendes et al., the greatest number of immunostained cells in the cochlea were type I fibrocytes in the spiral ligament. Moreover, we found KCTD8, 12, and 16 expressed in the spiral ganglia of the cochlea (Figure 4-9D-F). Concordantly, functional $GABA_B$ receptors were demonstrated by Lin et al. to be expressed in spiral ganglion neurons. These receive GABAergic innervation from the lateral olivocochlear efferent system, originating from neurons located around the lateral superior olivary nucleus (Lin et al., 2000). Thereupon Maison et al. showed that $GABA_B$ receptors are present only in type I and type II ganglion cells, their unmyelinated dendrites within the cochlea and their afferent terminals contacting inner and outer hair cells (Maison et al., 2009).

Apart from the cochlea, KCTD12 and/or KCTD16 are present in the main areas and nuclei that are significantly involved in the auditory pathway (Table 4-1). These comprise the superior olivary complex and the nucleus of the lateral lemniscus, which is known for its prominent GABA and glycinergic neurons. It also comprises the nucleus of the trapezoid body which expresses only KCTD12, and the medial geniculate nucleus which only expresses KCTD16 mRNA. Additionally, we found KCTDs expressed in the dorsal cochlea nucleus (DCN) through which auditory nerve fibers from the cochlea enter the brain. The DCN displays high levels of KCTD8 and 12, and low levels of KCTD16 transcripts (Figure 4-9J-L). As the DCN projects reciprocally to the inferior colliculus and also receives efferent innervation from the auditory cortex, and the superior olivary complex, it is thought to be involved in more complex auditory processing.

Superior and inferior colliculus also abundantly express $GABA_B$ receptors. Interestingly, both share low levels of KCTD12 mRNA in the adult brain, but high levels at P5. In contrast to mRNA levels of KCTD16, which are low postnatally, but very high during adulthood (for comparison see Table 4-1 and Table 4-2). The different expression levels between P5 and adult brain strongly suggest a role of KCTD12 and 16 in the development of visual and auditory pathways.

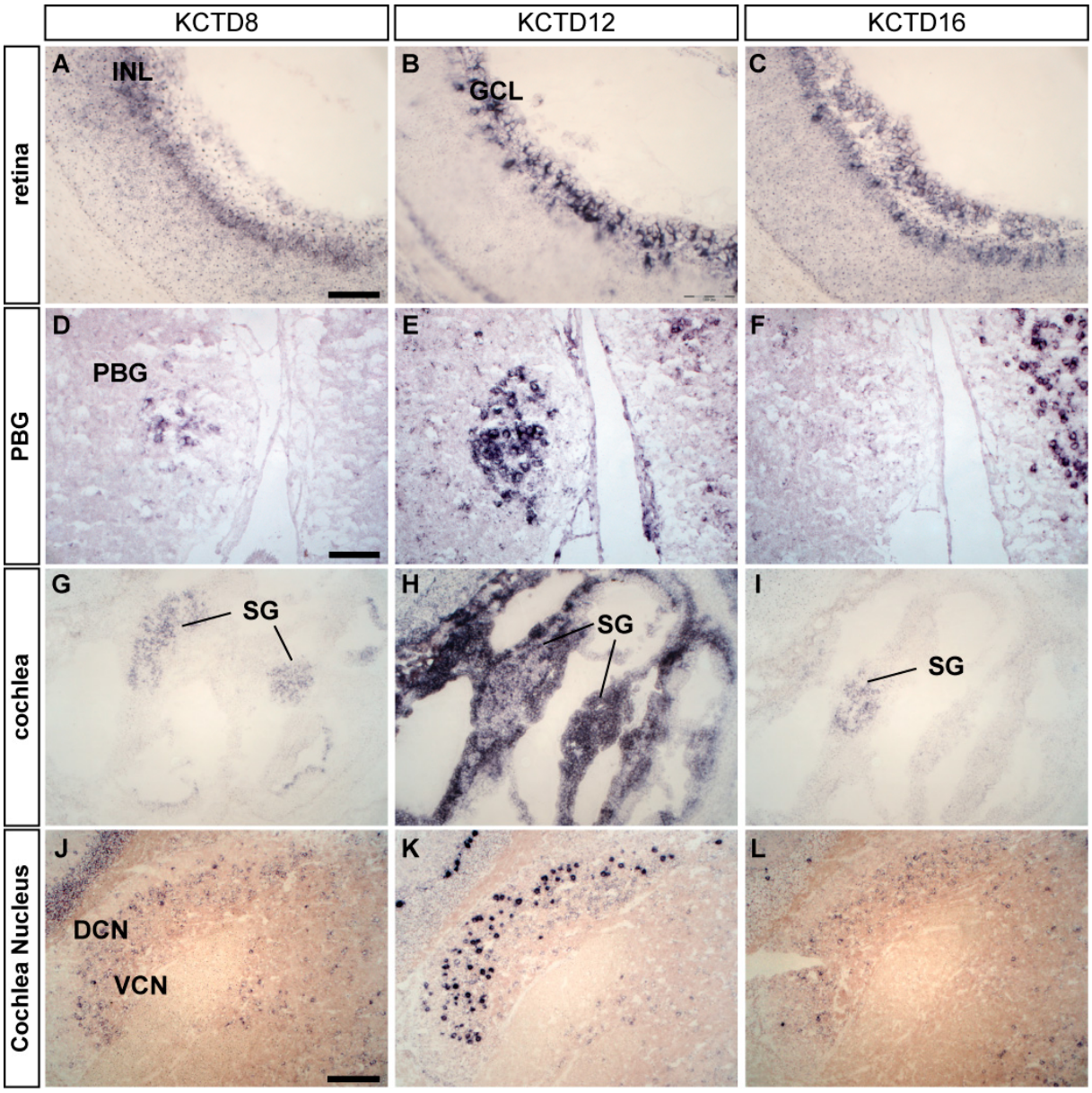

Figure 4-9: KCTD expression in the visual and auditory system. KCTD8 and 12 are expressed in the inner nuclear layer (INL), KCTD12 and 16 in the ganglion cell layer (GCL) of the retina (A-C). Neurons in the parabigeminal nucleus (PBG) express KCTD8 (D) and very high levels of KCTD12 mRNA (E). All KCTDs mRNA are expressed in the spiral ganglia (SG) of the cochlea (G-I), KCTD12 at very high levels (H). KCTD8 and high levels of KCTD12 were found in the dorsal cochlea nucleus (DCN) (J and K). Scale bars = 100μm (A-I), 200μm (J-L)

Sensorymotor system. Analogously, we observed KCTD8, 12, and 16 mRNA being present in structures belonging to the whisker sensorymotor system. KCTD8 and 16 are strongly expressed in the ganglion cells of the trigeminus (Figure 4-10A-C), whereas KCTD12 is individually expressed in whisker follicles (Figure 4-10D). In the whisker system, the cells of the trigeminal ganglion act as sensory receptor neurons. Nerve endings of the trigeminal ganglion convert mechanical energy sensed by mechanoreceptors in the whisker follicle into action potentials. We have shown KCTD12 and 16 being expressed in the ventral posterior

medial and the posterior nuclei of the thalamus, which receive input from the trigeminal nucleus and project to the barrel field of the somatosensory cortex. In addition to whisker follicles, high levels of KCTD12 were found in hair follicles (Figure 4-10E).

Apart from its function in the whisker sensorymotor system, the trigeminal ganglion contains a wide range of sensory and motor fibers, i.e. from the ophthalmic, maxillary, and mandibular nerves. Among others, the anti-nociceptive effect of the $GABA_B$ receptor agonist baclofen indicates a modulatory role of the $GABA_B$ receptor in processing information from the excitatory glutamatergic nociceptive primary afferents - the terminals of glutamatergic dorsal root and trigeminal ganglion cells. Like the mRNA of KCTD8 and 16, the $GABA_B$ receptor is present in the ganglion cells of the trigeminal. Unique and very high expression of KCTD12 was observed in the odontoblasts layer of the dental pulp of P5 animals (Figure 4-10F). GABAergic modulation of nociception was also here suggested by Todd et al. when they reported moderate GABA staining in the odontoblast layer and light staining within the subodontoblastic layer of pulpa and dentin which was increased in carious teeth (Todd et al., 1997). They proposed a role for GABA as peripheral neuromodulator or growth factor in the dental pulp and in inhibitory pulpal nerve mechanisms and neuroinhibition, causing the lack of symptoms in certain dental diseases. Expression of $GABA_B$ receptors in pulpa and dentin still awaits confirmation.

Among the other brainstem nuclei, moderate levels of KCTD12 were observed in the external cuneate nucleus, which receives fibers corresponding to the proprioceptive innervation of the upper-extremity, the pontine reticular nuclei, and the inferior olivary complex; all of them are involved in coordination of movement and project to the cerebellum. Notably the inferior olivary complex is the sole source of climbing fibers projecting to the cerebellum.

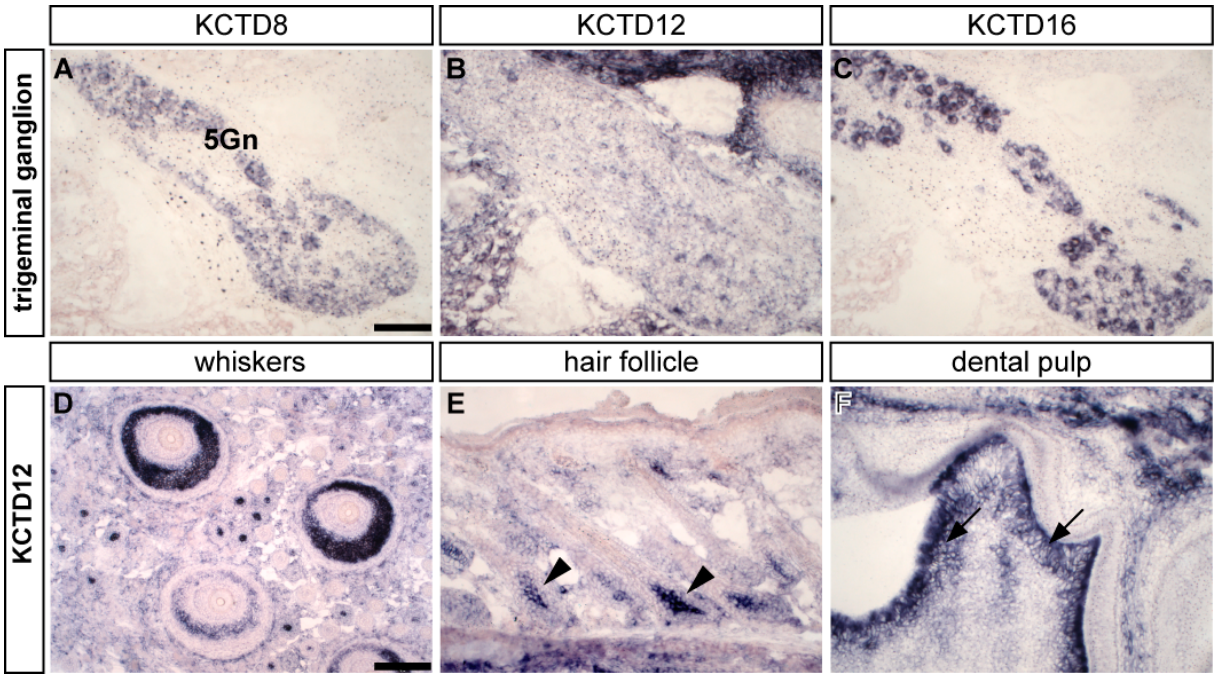

Figure 4-10: KCTD expression in the sensorymotor system. KCTD8 (A) and 16 (C) are highly expressed in the ganglion cells of the trigeminus nerve (5Gn). KCTD12 is strongly expressed in whisker follicles (D), hair follicles (E, arrowheads), and the odontoblasts layer of the dental pulp (F, arrows). Scale bars = 100μm (A–C), 200μm (D-F)

Cerebellum – distinct cellular KCTD expression pattern

While $GABA_B$ receptors are localized pre- and postsynaptically in both, GABAergic and glutamatergic cells of the cerebellum, the cerebellum shows very distinct expression patterns of KCTD8, KCTD12 and KCTD16 transcripts (Figure 4-11A-D). KCTD8 mRNA is expressed at moderate levels in the granule cell layer (GCL) (Figure 4-11B and F). Purkinje cells (PCs), which display the highest density of $GABA_B$ receptors in the cerebellum (Margeta-Mitrovic et al., 1999), also show the strongest KCTD12 mRNA levels Figure 4-11C, arrows). Throughout the cerebellum, PCs exhibit strikingly alternating stretches of labeled and non-labeled cells resembling the Zebrin II zonation pattern with higher density of KCTD12 positive Purkinje cells in the posterior than in the anterior part (Figure 4-11G). This pattern was also reported for the $GABA_B$ receptor (Fritschy et al., 1999; Lujan and Shigemoto, 2006) and nicely emphasizes the strong association between the $GABA_B$ receptor and the KCTDs. A high abundance of KCTD12 protein in PCs could ensure the presence of a high affinity type of $GABA_B$ receptor that is necessary for the enhanced long-term depression observed at parallel fiber - PC synapses (Kamikubo et al., 2007). KCTD16 mRNA is observed exclusively in large singularly scattered cells in the granular layer; the GABAergic Golgi cells (GC) (Figure 4-11D and H, arrowheads). In fact, also Golgi cell dendrites were reported to be

restricted to these Zebrin II positive Purkinje cell stripe boundaries (Sillitoe et al., 2008). The deep cerebellar nuclei contain low levels of KCTD8, 12, and 16 (Table 4-1).

The cerebellum is one of the few areas in the brain that develops during late embryonic and early postnatal stages. From P7 onwards, intense $GABA_{B1}$ immunoreactivity was reported in PCs, and moderate staining in the internal granular layer and the migrating granule cells (Lujan and Shigemoto, 2006). In contrast, KCTD12 is specifically present in the differentiating Purkinje cell layer and in the internal granular layer (igl), whilst KCTD8 is expressed in the external granular layer (egl) (Figure 4-11J and K). At P5 KCTD16 transcripts are predominantly expressed in the deep cerebellar nuclei (Table 4-2).

The Zebrin-like expression pattern which can be observed for KCTD12 in the adult cerebellum is not yet established at postnatal day 5. Most probably this occurs at later stages, as described for the $GABA_B$ receptor around P15 (Lujan and Shigemoto, 2006).

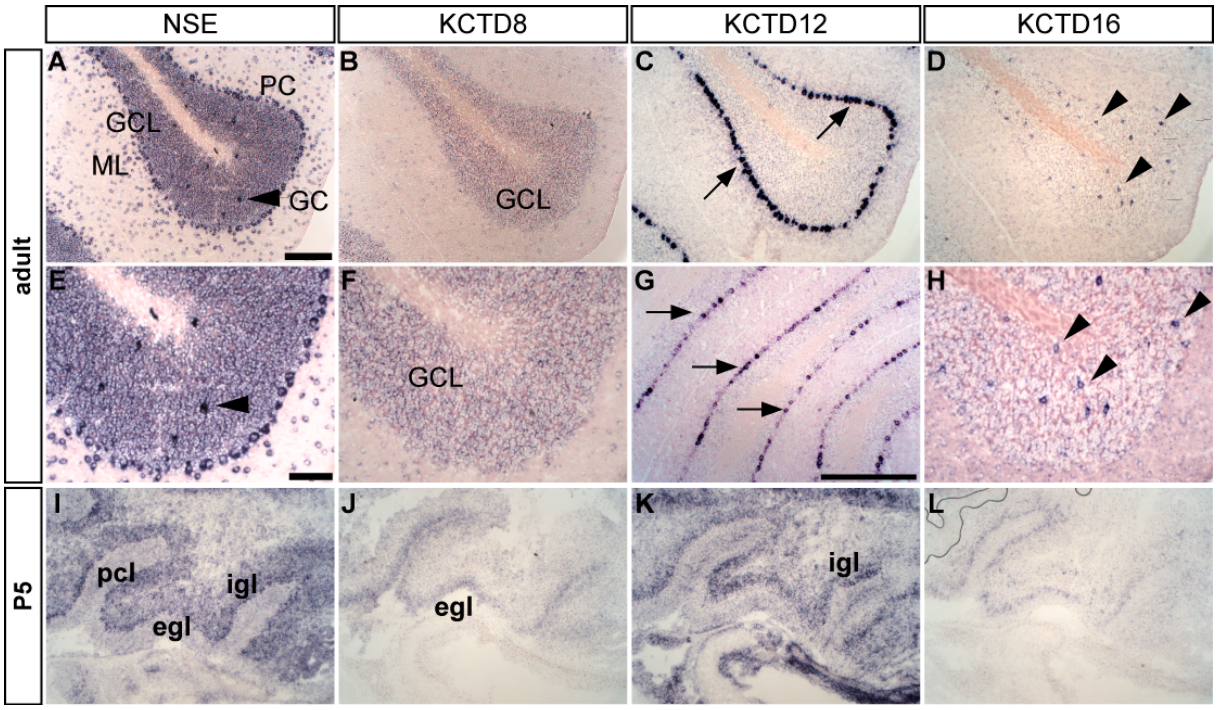

Figure 4-11: Distinct expression of KCTDs in the cerebellum. Granule cells (GCL) express KCTD8 (B, F), Purkinje cells (PC) KCTD12 (C, arrows), Golgi cells (GC) KCTD16 mRNA (D, H arrowheads). KCTD12 exhibits a Zebrin-like expression pattern in PCs (G, arrows). Hybridization of P5 brain sections reveals KCTD8 mRNA levels predominant in the external (egl) (J) and KCTD12 in the internal (igl) granular layers (K). GCL, granule cell layer; ML, molecular layer of the cerebellum. Scale bars = 200µm (A-D), 100µm (E, F and H-L), 1000µm (G).

Discussion

KCTD8, 12, and 16 were identified in immunoaffinity purification experiments using brain tissue as starting material and found to be tightly associated with the $GABA_B$ receptor (Schwenk et al., 2009). Furthermore Schwenk et al. described that these KCTDs differentially alter $GABA_B$ mediated activation of Kir- and Cav-channels. Thus the KCTDs may modulate various $GABA_BR$-mediated effects, and contribute to the diversity of $GABA_B$ receptor complexes with distinct functional properties.

Detailed information about the regional expression patterns of the KCTDs is required as a first step towards the understanding of their functional roles. We hypothesize that the differential subsets of KCTDs present in a certain $GABA_B$ receptor-signaling complex underlie the functional and pharmacological heterogeneity observed with native $GABA_B$ responses. They could act either alone or in combination, thereby conferring distinct properties onto the neurons in which they reside. The repertoire of KCTDs that can interact with $GABA_B$ receptors in a given neuron or brain area varies depending on the spatial and temporal expression patterns of KCTD8, 12, and 16. To address the role of KCTD8, 12, and 16 in the context of $GABA_B$ receptors we determined the expression patterns of KCTD transcripts in the brain, which revealed where individual KCTDs potentially interact with $GABA_B$ receptors. Our data demonstrate that the expression pattern of the $GABA_B$ interacting KCTDs overlap with those of the $GABA_B$ receptor (Bischoff et al., 1999; Durkin et al., 1999; Fritschy et al., 2004; Margeta-Mitrovic et al., 1999). $GABA_B$ receptor expression was detected throughout the brain, with highest expression in hippocampus, cerebellum, thalamus, and cerebral cortex. We found KCTD12 i.e. highly expressed in cerebellar Purkinje cells, a cell type that is known to containing high amounts of $GABA_B$ receptors. The observation that KCTD12 and $GABA_BR$ are both restricted to ZebrinII positive cells gives further support for a functional role of KCTD12 as auxiliary subunit of $GABA_B$.

Although highly related in sructure, the individual expression patterns of the KCTDs examined revealed striking differences. The expression of some KCTDs partially overlapped with each other in particular brain areas, such as the hippocampal formation, some nuclei of the amygdala or the cochlear nuclei; these are brain regions in which more than one member of the $GABA_B$ interacting KCTDs was identified. Other brain areas, like the nuclei of the anterior dorsal thalamus, the globus pallidus, the claustrum, the hypothalamic preoptic areas, and the Purkinje or Golgi cells of the cerebellum showed distinct non-overlapping KCTD expression patterns. Still, in most brain regions analyzed we identified several KCTDs, albeit expressed at different levels. KCTD12 and 16 are the most abundant $GABA_BR$ auxiliary subunits in the brain, whereas KCTD8 is specifically enriched in a very limited subset of cells, i.e., in the medial habenula, the cerebellar granule cells, and in several brain stem nuclei,

especially the cochlear nuclei, the spinal nucleus of trigeminal, and the hypoglossal nucleus. While KCTD16 exhibits a broad expression in structures like cortex, thalamus and amygdala, KCTD12 expression seems to be restricted to some nuclei or some subtypes of cells, but therein at very high levels.

Well known differences in the composition of $GABA_B$ receptor complexes arise from the two $GABA_{B1}$ isoforms $GABA_{B1a}$ and $GABA_{B1b}$. According to our data and previous localization studies (Bischoff et al., 1999; Durkin et al., 1999; Margeta-Mitrovic et al., 1999) cerebellar Purkinje cells would, i.e. exhibit a distinct $GABA_B$ receptor complex containing predominantly the $GABA_{B1b}$ subunit and KCTD12, exclusively. As $GABA_B$ receptors are localized postsynaptically at PC dendrites, KCTD12 might likely be involved in regulating $GABA_B$-mediated Kir responses at postsynaptic sites. In contrast $GABA_B$ receptor complexes in the mammillary body may contain predominantly the $GABA_{B1a}$ subunit and KCTD16. Quite a different $GABA_B$-KCTD complex is expected in the neurons of the medial habenula, which contain high amounts of $GABA_B$ receptors on their axonal processes (Bischoff et al., 1999; Margeta-Mitrovic et al., 1999). These $GABA_B$ receptor complexes are most likely composed of the $GABA_{B1b}$ subunit and KCTD8, 12 and 12b. Overall, these differences support our hypotheses that the $GABA_B$ interacting KCTD proteins display part of the heterogeneity of native $GABA_B$ receptors. Changes in $GABA_B$ subunit and $GABA_{B1}$ isoform expression during postnatal development (Bischoff et al., 1999; Fritschy et al., 2004) imply an important role of $GABA_B$ receptor complexes in the development of inhibitory neuronal circuits. Our data shows that KCTDs, according their expression profiles, may also contribute to these functions, especially in the establishment and function of sensory pathways.

A striking observation was the extensive and strong expression of KCTD12 in non-neuronal cells in the olfactory nerve layer, presumably in olfactory ensheathing cells. Olfactory ensheathing cells (OECs) are the principal glial cells of the olfactory bulb where axon growth not only occurs in response to injury but also occurs as a normal physiological process throughout normal life. OECs ensheath the axons within the olfactory nerve and constitute the major glial component of the superficially located nerve fiber layer of the olfactory bulb (Franklin and Barnett, 2000). Glial cells are known for the uptake and release of neurotransmitter and Jaffe et al. showed that GABA is released from glia cells on the olfactory bulb (Angulo et al, 2008; Jaffe and Cuello, 1981). Regarding the strong expression in OECs KCTD12 protein might likely be involved in this process. According to previous localization studies showing no expression of $GABA_B$ receptors in OECs, in this special case, $GABA_B$ receptors would not serve as interaction partners. However, $GABA_B$ receptors are expressed in several types of glial cells (Charles et al., 2003), and it might be assumed that

they regulate neuron-glial cross-talk in the nervous system (Magnaghi, 2007). Therefore re-examination of $GABA_B$ expression in OECs is needed.

In fact, immunolabelled $GABA_B$ receptors have been found in axon terminals of the olfactory nerve (Bonino et al., 1999), suggesting that activation of presynaptic $GABA_B$ receptors inhibits excitatory neurotransmission from olfactory nerve axons to second-order neurons. Apart from OECs we also observed KCTD12 mRNA in olfactory receptor neurons. A study by Priest and Puche revealed a function of $GABA_B$ receptors in olfactory axon growth. They showed that baclofen inhibits axon growth and suggested that GABA, which is often released prior to the formation of synaptic connections, might serve as a `stop and synapse` signal which acts on the $GABA_B$ receptors expressed by the ingrowing ORN growth cones (Priest and Puche, 2004). As olfactory sensory neurons are continuously regenerating neurons (Graziadei et al., 1979), this goes along with previous data suggesting a role of $GABA_B$ receptors in developmental events, such as neuronal migration (Behar et al., 2000; Behar et al., 1998; Lopez-Bendito et al., 2003), chemoattraction (Behar et al., 2001), and axonal pathfinding (Xiang et al., 2002).

Western blot analysis of two-dimensional gel separations of brain membrane fractions showed that all $GABA_B$ receptors resolved on native gels appeared to be associated with the KCTD proteins. Both KCTD12 and KCTD16 strictly co-migrated with the $GABA_B$ core subunits, but their signals exhibited only partial overlap with each other. This suggests that a portion of both KCTDs may be integrated into the same $GABA_B$ complexes, while the remaining KCTD proteins participate in distinct $GABA_B$ receptors of either higher (KCTD16) or lower (KCTD12) molecular size (Schwenk et al., 2009). Recently, Tiao et al. showed that the $GABA_B$ interacting KCTDs are able to homo- and heteromerize which supports the existence of heteromeric complexes (Tiao et al., 2009). We observed overlapping distribution in some brain areas, as well as very distinct localization for each $GABA_B$ interacting KCTD in other, strengthening their suggested capability to form distinct subtypes of $GABA_B$ receptor complexes. Whether $GABA_B$-mediated signaling is regulated differentially in neurons that express one individual KCTD or a certain subset of the $GABA_B$ interacting KCTDs still remains to be determined. For this the individual role of each $GABA_B$ interacting KCTD needs further examination; and moreover comprehensive investigation if KCTDs display synergistic or antagonizing effects is crucial.

However, it might be difficult to use the KCTD subset expression for predictions of the $GABA_B$-mediated signaling characteristics, as there are multiple other players involved in modulating the action of $GABA_B$. In regards to the K-channel signaling, different types of Kir-channels should be taken into account for possible heterogeneity of $GABA_B$ mediated Kir signaling (Aguado et al., 2008; Doupnik, 2008; Fernandez-Alacid et al., 2009). Some

members of the family of RGS proteins, for example, were also reported to modulate $GABA_B$-mediated Kir activity (Fowler et al., 2007; Labouebe et al., 2007).

In terms of function, it also has to be considered that the here explored distribution profile gives an instantaneous picture of the localization of the different KCTD mRNAs. If protein distribution exactly overlaps and if there are changes in protein levels under certain circumstances like increased neuronal activity or during formation of neuronal circuitries needs to be investigated. Localization studies of overexpressed KCTD proteins (Tiao et al., 2009) suggest the presence of KCTDs at presynaptic, as well as at postsynaptic sites. However, our data provides the basic for future studies which may elucidate preferences of the individual KCTDs in regulating pre- or postsynaptic $GABA_B$-mediated actions at certain circuitries, like we suggested for the cerebellum.

Conclusions

This is the first study that reports in detail the spatial distribution of the $GABA_B$ interacting KCTD genes in the mouse nervous system. Knowledge of the expression patterns of these genes provides yet another tool to elucidate the function of the KCTD subfamily and moreover to explain the functional heterogeneity observed in native $GABA_B$ receptors. We found that KCTD12 and 16 are present throughout most brain areas, but each KCTD showing a high degree of regional specialization. In most brain regions at least one member of the KCTD subfamily is present. $GABA_B$ receptors are abundant throughout the whole brain and Schwenk et al. showed that literally all native $GABA_B$ receptors are associated with either one or more $GABA_B$ interacting KCTD (Schwenk et al., 2009). Areas that exhibit very high amounts of $GABA_B$ receptor protein, such as the pyramidal layers of the hippocampus, several amygdaloid nuclei, certain nuclei of the thalamus, or the Purkinje cells of the cerebellum also show high mRNA levels of either one ore more KCTD. Our study revealed quite distinct expression patterns of each individual KCTD subunit that may contribute to the diversity of $GABA_B$ receptor responses observed in vivo and strongly support their role as auxiliary subunits of the $GABA_B$ receptor.

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5 General Discussion

Discussion

$GABA_B$ receptors are involved in the fine tuning of inhibitory synaptic transmission. Presynaptic $GABA_B$ receptors inhibit neurotransmitter release by down-regulation of high-voltage activated Ca^{2+} channels, whereas postsynaptic $GABA_B$ receptors decrease neuronal excitability by activating a prominent inwardly rectifying K^+ conductance that underlies the late inhibitory postsynaptic potentials. In physiological conditions, investigators have observed a large variability in the kinetics and pharmacology of $GABA_B$ receptors that are not present when expressed in recombinant systems. One of the main issues of $GABA_B$ research is to elucidate the causes that lead to the heterogeneity of native $GABA_B$ receptors. Identification of the $GABA_{B1}$ isoforms, 1a and 1b, was in line with the expectation that there exists a variety of distinct receptor subtypes as reported e.g. for the metabotropic glutamate receptors. But the absence of functional or pharmacological differences of $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ in vitro made clear that the two receptor subtypes do not account for the functional heterogeneity of native $GABA_B$ receptors. This suggests the existence of alternative factors that can modify receptor activity or pharmacology. To this end, our laboratory initiated a proteomics' based approach to screen for $GABA_B$ receptor interacting molecules. GPCRs have been shown to mediate signaling through large multiprotein complexes. Unexpectedly, there is so far no evidence for large protein complexes containing $GABA_B$ receptors. This is particularly surprising when one considers that macromolecular complexes have been described for classic effectors of $GABA_B$ receptors such as the voltage-dependent Ca^{2+} channel. In this particular instance, Ca^{2+} channels have been shown to function as adaptor proteins for multiple molecules that bind the channel and signal to downstream components following $GABA_B$ receptor activation (Richman et al., 2004). Possible reasons for the difficulty in identifying macromolecular complexes associated with $GABA_B$ receptors may lie within the receptor heterodimer itself. Most likely, the formation of a compact dimer between $GABA_{B1}$ and $GABA_{B2}$ results in the exposure of novel molecular surfaces for protein interactions, probably absent in the individual subunits, a factor that has hitherto been hindering identification of native $GABA_B$ receptor interactors. In addition, only a limited number of receptor domains have been used to search for specific interacting proteins, probably due to the low signal-to-noise ratio of some recombinant techniques. The search for components of a macromolecular complex involved in the direct modulation of $GABA_B$ receptor signaling may therefore require the use of new biochemical approaches using native $GABA_B$ receptor heterodimers. Using such a proteomic approach, we identified KCTD8, 12, 12b, and 16 as novel interacting proteins of the $GABA_B$ receptor. Subsequently

they were shown to be auxiliary subunits of the receptor which are integrated in native $GABA_B$ receptor complexes and profoundly alter the signaling properties of the receptor. KCTD proteins significantly enlarge the functional spectrum of $GABA_B$ receptors and have the potential to generate receptors with distinct properties. This dissertation describes the subtype-specific properties of the KCTDs and the distinct KCTD-$GABA_B$ complexes that are possible. Furthermore, we give evidence that the assembly of KCTDs in native $GABA_B$ receptor complexes may account for the differences in kinetics and pharmacology observed in native $GABA_B$ receptors.

Chapter two contains two main findings. First, we showed that native $GABA_B$ receptors exist, not as heterodimers as believed to date, but as heteromultimers. Second, we describe four novel proteins that are stably associated within this native receptor complex. We investigated the structural determinants and the putative stoichiometry of the $GABA_B$-KCTD complex, and present an expression profile of the KCTD proteins in the brain as well as their subcellular localization. Functionally, we examined the modulating effects on the main effector systems of the $GABA_B$ receptor, Kir and Cav channels.

Dynamically assembled macromolecular protein complexes that localize into discrete membrane microdomains resemble more and more the contemporary picture of GPCR and ion channel signaling and replace the traditional concept of GPCRs conceptualized as monomeric or dimeric proteins. Macromolecular protein complexes that transduce biological signals across the cell membrane have several advantages over their dissociated components functioning through random collisions. At first, the assembled complex provides a more rapid response time because the protein participants are not diffusion-limited or dependent on bulk concentration. Second, signaling complexes reduce noise or "cross-talk" that occurs with nonspecific collision events. And finally, they are metabolically more efficient because energy costs associated with the synthesis and maintenance of the localized protein complex are significantly lower than those required for collision-coupled signaling over the entire cell volume. The group of J. P. Pin has specialized in the investigation of GPCR oligomerization by the use of energy transfer technologies, such as fluorescence energy transfer (FRET) or bioluminescence energy transfer (BRET) combined with snap-tag technology and thus provided evidence that $GABA_B$ receptors at the cell surface assemble into higher order structures (Pin et al., 2009). We have now demonstrated that native $GABA_B$ receptors indeed form heteromultimeric complexes.

The results in chapter two suggest that KCTDs regulate the G-protein cycle upon activation of $GABA_B$ receptors. KCTD mediated effects require the direct interaction between the KCTD and the $GABA_{B2}$ subunit. KCTD12 and 16 were shown to increase agonist affinity, which is in agreement with the assumption that the KCTD action takes place at the receptor. For notion,

RGS proteins were not shown to alter agonist binding on any GPCRs. Similarly they are not found to bind directly to GPCRs. By resolving the onset and activation phase of the $GABA_B$-mediated Kir response we found that all KCTDs accelerate the $GABA_B$ response. This acceleration could be explained by an expedited activation of the effector channels due to a faster GDP/GTP exchange at the receptor bound $G\alpha_i$ which would suggest that KCTDs modulate guanine nucleotide exchange factor (GEF) activity of $GABA_B$ receptors. KCTD12 and 12b, but not KCTD8 and 16, showed a significant effect in receptor desensitization, which encouraged our hypotheses that the decrease of receptor activity during sustained agonist application is based on a subsequent transition of the receptor into an inactivated state that precludes further GDP/GTP exchange. This inactive state is presumably achieved by conformational changes in the receptor and/or the KCTDs. KCTD8 and 16 differ from KCTD12 and 12b in an additional c-terminal element, which makes conformational changes very comprehensible. $GABA_{B2}$-KCTD binding was not affected by agonist application in Co-IP as well as in FRET-interaction studies. Therefore, we consider the possibility of dissociation of KCTDs and $GABA_{B2}$ as unlikely. However, suggestions and hypotheses for mechanisms underlying the KCTD-mediated effects presented in this chapter remain to be verified.

The third chapter includes data showing that the KCTDs form homo- and heterodimers among themselves as well as with the $GABA_B$ receptor and puts emphasis on the structural requirements for these interactions. We thoroughly investigated the functions of the conserved KCTD domains in respect to dimerization and physiological properties. The ability of the KCTDs to simultaneously bind to themselves and the $GABA_B$ receptor via different domains provides the molecular prerequisites for the formation of differentially composed complexes. It also presents parallels to T1 domains of Kv channel subunits which were shown to mediate subunit specific assembly and thus serve as a control mechanism for correct channel forming. The observation that none of the two domains of KCTD8 is sufficient to bind to the receptor suggests the existence of further specific interaction properties of the KCTDs encoded by their amino acid sequence, their secondary or tertiary structure. The electrophysiological data presented in the third chapter go beyond the ones presented in the second, particularly in two points. On one hand, it identifies the domains, necessary for KCTD-KCTD and KCTD-$GABA_B$ interactions, which provide the means to form higher order structures composed of the $GABA_B$ receptor and its auxiliary subunits. Secondly, the experimental setup using hippocampal slice cultures closer resembles the physiological structures with preserved neuronal networks. The effects of KCTD overexpression are, compared to the ones observed in heterologous cells or dissociated neuronal cultures, less pronounced which suggest that more factors, like other binding partners or phosphorylation,

may add to the complexity of native $GABA_B$ receptor signaling. Similarly to chapter two, the underlying mechanisms, which most likely involve modulation of the G-protein cycle, remain to be elucidated.

In the fourth chapter we describe the spatial and temporal expression pattern of KCTD8, 12, and 16 in the CNS, examined by in situ hybridization. We show that the distribution of the KCTDs is widely overlapping with the hitherto described distribution of $GABA_B$ receptors. The study concentrates on the identification of brain areas which exhibit expression of distinct subsets of one ore more KCTDs. The overall KCTD mRNA levels are coherent with the affinity purification data presented in chapter two, showing KCTD12 and 16 being copurified with the $GABA_B$ receptor at high yields, whereas KCTD8 in slightly smaller and KCTD12b in markedly smaller amounts. Moreover the two dimensional gel separation (Figure 2-1d) suggested that KCTD12 and 16 are part of the same $GABA_B$ receptor complex, but additionally participate in distinct $GABA_B$ receptor complexes. These assumptions could be affirmed by the revealed expression patterns of the KCTDs, which are partially overlapping, but in wide ranges very distinct.

$GABA_B$ receptors mediate their actions throughout the whole CNS and are located at various cellular compartments. This is one of the reasons which makes the exploration of specific effects of the receptor difficult and also renders the specific therapeutic targeting of $GABA_B$ receptor mediated actions very demanding. It also underlines the importance to identify how variety in the $GABA_B$ system is achieved and its relevance for basic research and drug development.

Perspectives

One of the most interesting points for further research remains with the exact elucidation of the mechanism of KCTD action on the G-protein cycle. This question will be addressed by means of different GTPγS assays and BRET/ FRET studies, which are currently established in our laboratory. The dose-response curves described in chapter two have revealed that KCTD12 and 16 increase the apparent agonist affinity of $GABA_B$ receptors. Radioligand binding assays and [35S]-GTPγS binding assays will be performed to show how KCTDs exert this effects. A decrease of the EC_{50} determined by electrophysiology could result from a higher agonist affinity due to a conformational change of the receptor, an increase in cell surface receptors, or from a change in G-protein activation/deactivation. The agonist binding affinity of the receptor and the number of surface receptors can be measured by competitive and saturating radioligand binding assays, respectively. Furthermore, in [35S]-GTPγS binding assays the effects on the activation rate is determined by measuring the binding of the non-

hydrolyzable GTP analogue, [35S]-GTPγS, bound to Gα, which is the first step of the G-protein cycle. FRET/ BRET studies will allow us to investigate if KCTDs have effects on the dissociation and re-association rate of the Gα with the Gβ and Gγ subunits, or of the Gα with the $GABA_B$ receptor. Affinity purifications showed that KCTDs interact with Gαo, Gβ, several subunits of Cav and Kir channels. BRET assays will be used to examine which of these are direct interactions. Importantly, we will test whether KCTDs induce a conformational change in the $GABA_B$ receptor by means of homogeneous time-resolved FRET, where the interaction between the two receptor subunits will be measured in presence and absence of KCTDs. These assays will enable us to identify and discriminate between possible KCTD-mediated effects.

In regards to the homo- and heteromeric assembly of KCTDs and $GABA_B$ receptors it would be interesting to know if KCTDs compete in the binding of $GABA_B$ receptors or if a certain composition is favored. In native receptors the composition of the complex is predetermined by the KCTD subset expressed by a neuron, but could in addition be conditioned by different affinities or could involve other binding partners.

Our laboratory has made large efforts to generate specific knockout animals for KCTD8, 12, 12b, and 16. These animals will soon provide very useful tools to further investigate the functions and the underlying mechanism of KCTD actions. In addition they will give hints on the main physiological impacts of the KCTDs. In spite of the fact that the knockout animals can serve as controls in various analyses, in detail there are several experiments pending. In regards to the localization studies we will add an immunohisto-/cytochemical approach to identify subsets of KCTDs in certain cell types, like certain glutamatergic or cholinergic neurons, or subpopulations of GABAergic interneurons. As we are so far not able to confidently exclude a cross reactivity of our specific KCTD antibodies in tissue or cell culture preparations, we aim to validate existing data by taking advantage of knockout animals. Knowledge of the KCTD subset present in certain cell types would allow us to investigate and compare their $GABA_B$ receptor modulating properties and moreover relate them to the known characteristics of the certain cell populations, e.g. expression of certain GPCRs or Kir subtypes. The distribution data identified brain structures that strongly express KCTDs and might show strongest defects in the knockout animals. Therefore these data are important to predict changes in potentially affected structures or behavioral skills that are connected with them. Certainly the data might help to understand possible phenotypes.

For the electrophysiological characterization of the knockout animals this analysis gives us the ability to target specifically cells that express only one single KCTD, which should make it more feasible to identify effects mediated by the certain KCTDs. In terms of function, we

would like to closer define the electrophysiological properties of different KCTD-$GABA_B$ combinations in heterologous and neuronal cells, respectively and later on compare them with the corresponding complexes in vivo.

A major goal is the identification of KCTD interaction partners, which will help to shed light on the mechanisms and signaling pathways through which KCTDs mediate their actions and might open up new concepts of the functions of KCTD proteins. In particular, novel interaction partners of KCTD12 could give hints to the role of KCTD12 in neuronal development. As KCTDs are part of a $GABA_B$ receptor complex and also form dimers and heterodimers by themselves, the search for putative interaction partners will encounter similar difficulties as described previously for $GABA_B$ receptor heterodimers. Therefore we will again focus on the proteomic approach in collaboration with Bernd Fakler, similar to the reverse affinity purifications with specific KCTD antibodies described in chapter two. Being able to acquire comparable data from knockout animals will add further specificity and value to these experiments. In general, the null-mutant animals should clarify, if KCTDs are essential for $GABA_B$ receptor-mediated functions. They should display putative impacts of KCTDs in brain development and function. However, it would not be surprising, if KCTD knockout animals show only very mild impairments. The redundancy among different KCTDs might complicate the analysis of KCTD function, which would make it necessary to generate double/ triple knockouts to circumvent compensatory effects.

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