The Sushi Domains of GABA_B Receptors Function as Axonal Targeting Signals

Barbara Biermann,1,2* Klara Ivankova-Susankova,1* Amyaouch Bradaia,1 Said Abdel Aziz,1 Valerie Besseyrias,1 Josep P. Kapfhammer,1 Markus Missler,2 Martin Gassmann,1 and Bernhard Bettler1

1Department of Biomedicine, Institute of Physiology, Pharmazentrum, University of Basel, CH-4056 Basel, Switzerland, 2Institute of Anatomy and Molecular Neurobiology, Westfälische Wilhelms-University, D-48149 Münster, Germany, and 3Department of Biomedicine, Institute of Anatomy, University of Basel, CH-4056 Basel, Switzerland

GABA_B receptors are the G-protein-coupled receptors for GABA, the main inhibitory neurotransmitter in the brain. Two receptor subtypes, GABA_B1a and GABA_B1b, are formed by the assembly of GABA_B1a and GABA_B1b subunits with GABA_B2 subunits. The GABA_B1a subunit is a shorter isoform of the GABA_B1a subunit lacking two N-terminal protein interaction motifs, the sushi domains. Selectively GABA_B1a protein traffics into the axons of glutamatergic neurons, whereas both the GABA_B1a and GABA_B1b proteins traffic into the dendrites. The mechanism(s) and targeting signal(s) responsible for the selective trafficking of GABA_B1a protein into axons are unknown. Here, we provide evidence that the sushi domains are axonal targeting signals that redirect GABA_B1a protein from its default dendritic localization to axons. Specifically, we show that mutations in the sushi domains preventing protein interactions preclude axonal localization of GABA_B1a. When fused to CD8, the sushi domains polarize this uniformly distributed protein to axons. Likewise, when fused to mGlur1a the sushi domains redirect this somatodendritic protein to axons. The mechanism(s) and targeting signal(s) responsible for the selective trafficking of GABA_B1a into axons are unknown. Here, we provide evidence that the sushi domains are axonal targeting signals that redirect GABA_B1a protein from its default dendritic localization to axons. Specifically, we show that mutations in the sushi domains preventing protein interactions preclude axonal localization of GABA_B1a. When fused to CD8α, the sushi domains polarize this uniformly distributed protein to axons. Likewise, when fused to mGlur1a the sushi domains redirect this somatodendritic protein to axons, showing that the sushi domains can override dendritic targeting information in a heterologous protein. Cell surface expression of the sushi domains is not required for axonal localization of GABA_B1a. Altogether, our findings are consistent with the sushi domains functioning as axonal targeting signals by interacting with axonally bound proteins along intracellular sorting pathways. Our data provide a mechanistic explanation for the selective trafficking of GABA_B1a receptors into axons while at the same time identifying a well-defined axonal delivery module that can be used as an experimental tool.

Introduction

GABA_B receptors exert distinct regulatory effects on synaptic transmission (Couve et al., 2000; Bowery et al., 2002; Ulrich and Bettler, 2007). Presynaptic GABA_B receptors inhibit the release of GABA (autoreceptors) and post-synaptic GABA_B receptors inhibit neuronal excitability by activating K⁺ channels. Receptor subtypes are based on the subunit isoforms GABA_B1a and GABA_B1b, both of which combine with GABA_B2 subunits to form two heteromeric receptors, GABA_B1a2 and GABA_B1b2 (Marshall et al., 1999). Most if not all neurons in the CNS express GABA_B1a2 and GABA_B1b2 receptors. The GABA_B1a and GABA_B1b subunit isoforms derive from the same gene by alternative promoter usage and solely differ in their N-terminal ectodomains (Kaupmann et al., 1997; Steiger et al., 2004). GABA_B1a contains at its N terminus two sushi domains (SDs) that are lacking in GABA_B1b (Hawrot et al., 1998). SDs, also known as complement control protein (CCP) modules or short consensus repeats (SCR), are conserved protein interaction motifs present in proteins of the complement system, in adhesion molecules and in G-protein-coupled receptors (Morley and Campbell, 1984; Kirkitadze and Barlow, 2001; Grace et al., 2004; Lehtinen et al., 2004; Perrin et al., 2006). The tertiary structure of SDs is fixed by two intramolecular disulfide bridges that are critical for interaction with other proteins (Soares and Barlow, 2005). Consistent with their role as interaction motifs, the SDs of GABA_B1a recognize binding sites in neuronal membranes (Tiao et al., 2008).

The individual functions of the GABA_B1a and GABA_B1b subunit isoforms were dissected by comparing genetically modified 1a−/− and 1b−/− mice, which express either one or the other isoform (Pérez-Garcí et al., 2006; Shaban et al., 2006; Vigot et al., 2006; Ulrich and Bettler, 2007; Ulrich et al., 2007; Guetg et al., 2009). It was found that only GABA_B1a receptors inhibit glutamate release in response to endogenous GABA, while both GABA_B1a2 and GABA_B1b2 receptors mediate postsynaptic inhibition. This is a consequence of a selective trafficking of GABA_B1a receptors into axons. Specifically, experiments with organotypic slice cultures revealed that heterologously expressed GABA_B1a subunits traffic to axons and dendrites, while GABA_B1b subunits traffic to dendrites only (Vigot et al., 2006). The signals and mechanisms leading to a somatodendritic expression of GABA_B1b subunits and a more uniform distribution of GABA_B1a are unknown.
subunits are unknown. In general, polarized sorting of transmembrane proteins relies on signals in the targeted protein themselves (Craig and Banker, 1994; Winckler and Mellman, 1999). Since the targeting location of the shorter GABA$_{B1a,2}$ receptor is the somatodendritic compartment, this suggests that the longer GABA$_{B1a,2}$ receptor also contains common dendritic targeting signals in either the GABA$_{B1a}$ or the associated GABA$_{B1b}$ subunit. This implies a mechanism that prevents a fraction of GABA$_{B1a,2}$ receptors from trafficking to the default somatodendritic compartment and instead directs them to axons.

Here, we report that GABA$_{B1a,2}$ receptors are trafficked into axons by the SDs, which function as axonal targeting signals along intracellular sorting pathways. We discuss the mechanistic and regulatory implications of our findings.

Materials and Methods

**Mouse strains.** Primary neuronal cultures were prepared from WT BALB/c mice or 1a$^{-/-}$, 1b$^{-/-}$, and 2$^{-/-}$ mice that were strictly kept in the BALB/c inbred background (Schuler et al., 2001; Gassmann et al., 2004; Vigo et al., 2006). All animal experiments were subjected to institutional review and conducted in accordance with Swiss guidelines and approved by the veterinary office of Basel-Stadt.

**Generation of mutant proteins.** Cloning of Myc-tagged expression constructs was based on a strategy described earlier (Fagano et al., 2001). Briefly, to allow detection of transiently expressed subunits, the intrinsic signal peptides were replaced by 36 residues encoding the mGluR5 signal peptide (MVLLILVSLIKEDVQSAQ; followed by the Myc-tag). The TREQKLIQSEEDLRT replaced residues: Myc-GB1a, 1-16 (Kaupmann et al., 1997); Myc-GB1b, 1-29 (Kapmann et al., 1997); Myc-mGluR1a, 1-21 (Masu et al., 1991); Myc-CD8a, 1-21). The mGluR5 signal peptide was used because it is known to accurately release N-terminal epitope tags (Ango et al., 1999). To generate Myc-GB1aCS, the four cysteine residues of GABA$_{B1a}$ at positions 92, 95, 96, and 156 (Kapmann et al., 1997) were mutated to serine residues by site-directed mutagenesis of thymine to adenine. To generate Myc-GB1aASD1 and Myc-GB1aASD2, residues G$^{28}$ to C$^{199}$ or V$^{30}$ to Q$^{157}$ of Myc-GB1a were deleted. To generate Myc-SDs-mGluR1a, residues G$^{1}$ to T$^{134}$ of GABA$_{B1a}$ were introduced after the Myc-tag in rat Myc-mGluR1a (mGluR1a was a gift from R. M. Duvoisin, Oregon Health and Science University, Portland, OR). To generate Myc-SDs-CD8a, the residues G$^{1}$ to T$^{134}$ of GABA$_{B1a}$ were introduced after the Myc-tag in Myc-CD8a (CD8a was a gift from G. A. Banker, Oregon Health and Science University, Portland, OR). Initially, all constructs were subcloned into the cytomegalovirus-based eukaryotic expression vector pCI (Promega) to confirm protein expression in HEK293 cells. Subsequently all constructs were shuttled into plasmid pMH-SYN-1 for expression under control of the synapsin-1 promoter in cultured hippocampal neurons (gift from T. G. Oertner (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) and K. Svoboda (Howard Hughes Medical Institute, Ashburn, VA)). In GB1a-GFP and GB1b-GFP, the coding sequence for GFP was cloned in frame at the C terminus of full-length GABA$_{B1a}$ and GABA$_{B1b}$ (Kaupmann et al., 1997), leaving the cognate signal peptides unaltered. All constructs were verified by sequencing.

**Neuronal culture and transfection.** Cultured hippocampal neurons were prepared as described previously (Brewer et al., 1993; Goslin et al., 1998). Briefly, embryonic day 16.5 mouse hippocampi were dissected, digested with 0.25% trypsin in Hank’s solution (Invitrogen) for 15 min at 37°C, dissociated by trituration, and plated on glass coverslips coated with 1 mg/ml poly-l-lysine hydrobromide (Sigma) in 0.1% bovine (bovine serum; Sigma). Brains were dissected with 1,000 units/ml Pen/Strep (penicillin G and streptomycin sulfate, Sigma); 100 µg/ml Pen/Strep. For the first 4 h to allow neurons to attach. Subsequently, the coverslips were transferred to a feeder layer of primary astrocytes in serum-free medium [1X MEM with Glutamax, 0.3% glucose (w/v), and 1% Pen/Strep] supplemented with 1% N2 (Invitrogen). Primary astrocytes were obtained from newborn P0-P1 BALB/c mice. To prevent extensive proliferation of astrocytes 5 µM arabinoside (araC, Sigma) was added to the culture medium after 2 d. High-density cultures were grown in Neurobasal medium supplemented with B27 (Invitrogen), 0.5 mM l-glutamine, and 500 µg/ml Pen/Strep. In addition, 25 µM glutamic acid was added to the medium for the first 3 d. At DIV5, neurons were cotransfected with the appropriate expression constructs and soluble RFP (pMH-SYN-1, gift from R. Tsien, University of California San Diego, La Jolla, CA) using Lipofectamine 2000 transfection reagent (Invitrogen).

**Electrophysiology.** Hippocampal neurons were cultured for 2-3 weeks. On the day of the experiment, coverslips were placed in an interface chamber containing saline solution (140 mM NaCl, 3 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgCl$_2$, 11.1 mM glucose, 10 mM HEPES, pH 7.2) equilibrated with 95% O$_2$/5% CO$_2$ at 30–32°C. Neurons were visualized using infrared and differential interference contrast optics. Whole-cell patch-clamp recordings were performed at ~60 mV from the somata of neurons to measure mEPSCs in the presence of tetrodotoxin (1 µM) and bicuculline (10 µM). Patch electrodes (~3 MΩ) were filled with a solution containing the following: 140 mM Cs-gluconate, 10 mM HEPES, 10 mM phosphocreatine, 5 mM QX-314, 4 mM Mg-ATP, 0.3 mM Na-GTP, at pH 7.2 with Cs-OH and 285 mOsm. During the experiment drugs were applied by superfusion into the recording chamber. GABA$_{B}$ receptors were activated by baclofen (100 µM) and inactivated by the selective antagonist CGP34626 (1 µM). Detection and analysis of mEPSCs was performed by MiniAnalysis software (version 6.0.4, Synaptosoft). Experiments with CHO cells expressing WT or mutant GABAB receptors together with Kir3.1/3.2 channels and EGFP (used as a transfection marker) were performed at room temperature (RT) 2 d after transfection with Lipofectamine 2000 (Invitrogen). As a negative control, CHO cells expressing Kir3.1/3.2 channels and EGFP in the absence of GABA$_{B}$ receptors were used. Cells were continuously superfused with an extracellular solution composed of the following (in mM): 145 NaCl, 2.5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 25 glucose; pH 7.3, 323 mOsm. Patch pipettes were filled with an intracellular solution composed of the following (in mM): 107.5 potassium glutamate, 32.5 KCl, 10 HEPES, 5 EGTA, 4 MgATP, 0.6 Na-GTP, 10 Tris phosphocreatine; pH 7.2, 297 mOsm. GABA$_{B}$ responses were evoked by application of baclofen (10 s) (Dittet et al., 2006) and recorded with an Axopatch 200B patch-clamp amplifier. The presence of Kir3.1/3.2 channels in transfected cells was confirmed in voltage ramps from ~150 mV to +30 mV in the presence of a high extracellular potassium concentration (40 mM).

**Immunocytochemistry.** Neurons were fixed at DIV14 in 4% PFA/120 mM sucrose/PBS (137 mM NaCl, 8.5 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 3.0 mM KCl) for 20 min at RT, permeabilized with 0.25% Triton X-100 for 10 min and blocked for 1 h with 10% normal goat serum (NGS) in PBS. Primary antibodies were diluted in 10% NGS/PBS and incubated overnight at 4°C. After washing with 1X PBS, neurons were incubated with secondary antibodies diluted in 1% NGS/PBS for 1 h at RT. Primary antibodies were as follows: chicken anti-MAP2 (1:10,000; Abcam), rabbit anti-GABA$_{B1a}$-C-term [1:500; Clone B17 (Kulik et al., 2002); gift from R. Shigemoto (National Institute of Physiological Sciences, Okazaki, Japan)], mouse anti-β-tubulin (1:400; Sigma), mouse anti-Myc (1:500; Roche). Secondary antibodies were as follows: Alexa goat anti-chicken 647, Alexa goat anti-rabbit 568, and Alexa goat anti-mouse 488 (1:1,500; Molecular Probes). Neurons were imaged in: 15% PVA (Celvol polyvinyl alcohol Celanese Chemicals), 33% glycerol, and 0.1% sodium azide in PBS, pH 7–7.4. For imaging of transfected neurons, cultures were viewed on a Leica DM5000B fluorescence microscope. Glutamatergic neurons were discriminated from GABAergic neurons by their extensively branched spiny dendrites visualized by the RFP filling (Benson et al., 1994; Obersair et al., 2003). Digital pictures were captured using Soft Imaging System and AnalySIS software (F-View) and identically processed with Adobe Photoshop (RGB input levels, brightness/contrast).
the axonal versus dendritic distribution of heterologously expressed nolabeling was normalized to the fluorescence intensity of tubulin immunolabeling. The A:D ratio of GABAB1 protein is significantly different between WT and GABAB1(-/-) mice. Ac- tivation of GABAB heteroreceptors by baclofen, a GABAB receptor agonist, inhibits the spontaneous release of glutamate and as a result reduces the miniature EPSC (mEPSC) frequency (Yamada et al., 1999; Tiao et al., 2008). We found that baclofen strongly reduced the mEPSC frequencies in wild-type (WT) and 1b(-/-) mouse hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). Specifically, we addressed whether functional GABAB heteroreceptors are present in cultured pyramidal neurons of 1a(-/-) mice, while baclofen only marginally reduced the mEPSC frequency in 1a(-/-) neurons (Fig. 1A, B). This confirms that functional GABAB1 heteroreceptors are specifically lacking in cultured hippocampal neurons of 1a(-/-) mice. Weak residual heteroreceptor activity in 1a(-/-) mice in response to high concentrations of baclofen was also observed in acute hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). This may reflect that low amounts of GABAB1(-/-) heteroreceptors are present at glutamatergic terminals. Alternatively, baclofen

from Clontech. Pictures were taken with each filter separately. Pictures from the endogenous GABA<sub>B<sub>1</sub><sub>1</sub></sub> staining were captured using immersion oil without autofluorescence (Leica Microsystems catalog #11513859) and a 63× objective with 1.32 NA (HCX PL APO). Images to evaluate the axonal versus dendritic distribution of heterologously expressed GABA<sub>B<sub>1</sub></sub> protein were captured using a 20× air objective 0.7 NA (HC PLAN APO).

Quantification of axonal versus dendritic distribution. The axon-to-dendrite (A:D) ratio of endogenous GABA<sub>B<sub>1</sub></sub> protein was determined using MetaMorph Imaging software. One-pixel-wide lines were traced along representative axons and dendrites in the tubulin-stained images. Next to each line, a rectangle was drawn for background subtraction. Subsequently, the lines and rectangles were transferred to the corresponding picture along representative axons and dendrites in the tubulin-stained images.

Figure 1. Endogenous GABA<sub>B<sub>1</sub></sub> but not GABA<sub>B<sub>1</sub></sub> subunits inhibit glutamate release and localize to axons in cultured hippocampal neurons

Pyramidal neurons typically make up 85–90% of neurons in dissociated hippocampal cultures (Goslin et al., 1998) and potentially provide a simple experimental system to study the targeting of transfected GABA<sub>B<sub>1</sub></sub> and GABA<sub>B<sub>1</sub></sub> subunits in glutamatergic neurons. We first investigated whether cultured pyramidal neurons preserve the selective association of GABA<sub>B<sub>1</sub></sub> receptors with glutamatergic terminals seen in hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). Specifically, we addressed whether functional GABA<sub>B<sub>1</sub></sub> heteroreceptors are present in cultured pyramidal neurons of 1b(-/-) mice, but absent in neurons of 1a(-/-) mice. Activation of GABA<sub>B<sub>1</sub></sub> heteroreceptors by baclofen, a GABA<sub>B<sub>1</sub></sub> receptor agonist, inhibits the spontaneous release of glutamate and as a result reduces the miniature EPSC (mEPSC) frequency (Yamada et al., 1999; Tiao et al., 2008). We found that baclofen strongly reduced the mEPSC frequencies in wild-type (WT) and 1b(-/-) neurons, while baclofen only marginally reduced the mEPSC frequency in 1a(-/-) neurons (Fig. 1A, B). This confirms that functional GABA<sub>B<sub>1</sub></sub> heteroreceptors are specifically lacking in cultured hippocampal neurons of 1a(-/-) mice. Weak residual heteroreceptor activity in 1a(-/-) mice in response to high concentrations of baclofen was also observed in acute hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). This may reflect that low amounts of GABA<sub>B<sub>1</sub></sub>(-/-) heteroreceptors are present at glutamatergic terminals. Alternatively, baclofen

---

**Results**

Endogenous GABA<sub>B<sub>1</sub></sub> but not GABA<sub>B<sub>1</sub></sub> subunits inhibit glutamate release and localize to axons in cultured hippocampal neurons

Pyramidal neurons typically make up 85–90% of neurons in dissociated hippocampal cultures (Goslin et al., 1998) and potentially provide a simple experimental system to study the targeting of transfected GABA<sub>B<sub>1</sub></sub> and GABA<sub>B<sub>1</sub></sub> subunits in glutamatergic neurons. We first investigated whether cultured pyramidal neurons preserve the selective association of GABA<sub>B<sub>1</sub></sub> receptors with glutamatergic terminals seen in hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). Specifically, we addressed whether functional GABA<sub>B<sub>1</sub></sub> heteroreceptors are present in cultured pyramidal neurons of 1b(-/-) mice, but absent in neurons of 1a(-/-) mice. Activation of GABA<sub>B<sub>1</sub></sub> heteroreceptors by baclofen, a GABA<sub>B<sub>1</sub></sub> receptor agonist, inhibits the spontaneous release of glutamate and as a result reduces the miniature EPSC (mEPSC) frequency (Yamada et al., 1999; Tiao et al., 2008). We found that baclofen strongly reduced the mEPSC frequencies in wild-type (WT) and 1b(-/-) neurons, while baclofen only marginally reduced the mEPSC frequency in 1a(-/-) neurons (Fig. 1A, B). This confirms that functional GABA<sub>B<sub>1</sub></sub> heteroreceptors are specifically lacking in cultured hippocampal neurons of 1a(-/-) mice. Weak residual heteroreceptor activity in 1a(-/-) mice in response to high concentrations of baclofen was also observed in acute hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). This may reflect that low amounts of GABA<sub>B<sub>1</sub></sub>(-/-) heteroreceptors are present at glutamatergic terminals. Alternatively, baclofen
may also activate somatic GABA$_{B1a,2}$ receptors and the ensuing hyperpolarizing potentials passively propagate to glutamatergic terminals, where they contribute to presynaptic inhibition (Alle and Geiger, 2006).

We next analyzed the expression levels of the endogenous GABA$_{B1a}$ and GABA$_{B1b}$ proteins in axons and dendrites of cultured hippocampal neurons. Due to the lack of GABA$_{B1a}$- or GABA$_{B1b}$-specific antibodies, we used cultured hippocampal neurons from $1a^{-/-}$ and $1b^{-/-}$ mice and stained them with an antibody recognizing the common C-term of GABA$_{B1}$ subunits (Kulik et al., 2002). To distinguish dendrites from axons, we immunolabeled the dendritic microtubule-associated protein MAP2 and tubulin, a constituent of axons and dendrites (Caceres et al., 1984).

In WT and $1b^{-/-}$ pyramidal neurons, GABA$_{B1}$ immunostaining was observed in MAP2-positive somata and dendrites as well as in MAP2-negative axons (Fig. 1C). In contrast, in cultured $1a^{-/-}$ pyramidal neurons, GABA$_{B1}$ immunostaining was restricted to the somatodendritic compartment. This confirms that primarily GABA$_{B1a}$ localizes to axons in cultured pyramidal neurons. To determine the axon-to-dendrite (A:D) ratio of the endogenous GABA$_{B1}$ proteins, we normalized the red fluorescence intensity of the GABA$_{B1}$ staining to the green fluorescence intensity of the tubulin staining in axons and dendrites. In all three genotypes the A:D ratio was < 1, indicating that most GABA$_{B1a}$ protein is localized somatodendritically (WT: $0.54 \pm 0.05$, $n = 7$; $1a^{-/-}$: $0.22 \pm 0.02$, $n = 7$; $1b^{-/-}$: $0.60 \pm 0.05$, $n = 8$; $p < 0.001$ for $1a^{-/-}$ vs WT and $1b^{-/-}$). However, the A:D ratio in $1a^{-/-}$ neurons was significantly reduced compared to WT and $1b^{-/-}$ neurons (Fig. 1D), indicating that significantly more GABA$_{B1a}$ than GABA$_{B1b}$ protein enters the axonal compartment. In summary, our electrophysiological and immunocytochemical analysis demonstrates that cultured pyramidal neurons preserve the preferential association of GABA$_{B1a}$ with glutamatergic terminals seen in hippocampal slices (Fig. 1E).

Exogenous GABA$_{B1a}$ and GABA$_{B1b}$ subunits reproduce the distribution patterns of the endogenous subunits

We next assessed whether GABA$_{B1}$ isoforms with an N-terminal Myc-tag (Myc-GB1a, Myc-GB1b) recapitulate the subcellular distribution of the endogenous proteins when expressed in cultured hippocampal neurons. Cultured hippocampal neurons were transfected after 5 d in vitro (DIV5) with Myc-GB1a or Myc-GB1b cDNAs under control of the neuron-specific synapsin-1 promoter (Kügler et al., 2001; Boulos et al., 2006), as this promoter avoids randomization of distribution patterns due to overexpression (Vigot et al., 2006). To accurately release the N-terminal Myc-epitope in the Myc-GB1a and Myc-GB1b proteins, we used a surrogate signal peptide instead of the intrinsic signal peptides (Anigo et al., 1999). We coexpressed Myc-GB1a or Myc-GB1b with the freely diffusible red fluorescent protein (RFP) tdimer2, which outlines the morphology of the

Figure 2. Exogenous GABA$_{B1a}$ but not GABA$_{B1b}$ protein localizes to the axons of transfected hippocampal neurons in culture. A. Scheme of the tagged GABA$_{B1}$ isoforms (top). The gray bar indicates the two SDs (SD1, SD2) in GABA$_{B1a}$, the green bar the transmembrane domains. Myc-GB1a and Myc-GB1b cDNA expression constructs were individually cotransfected with a cDNA expression construct encoding soluble RFP. Neurons were fixed at DIV14, permeabilized, and stained with antibodies recognizing MAP2 (data not shown) or the Myc-tag. Low-magnification images of the merged green Myc and the RFP fluorescence are shown at the top. Higher-magnification images of the boxed regions depict axons (arrows) and dendrites (arrowheads). Scale bars: top, 50 $\mu$m; bottom, 10 $\mu$m. B. When analyzing the total Myc-GB1a and Myc-GB1b levels in transfected neurons (Total), the A:D ratio of Myc-GB1a is significantly higher than that of Myc-GB1b (mean $\pm$ SEM, **$p < 0.01$, Student’s $t$ test). Likewise, when analyzing Myc-GB1a and Myc-GB1b at the cell surface of neurons coexpressing exogenous GABA$_{B2}$ (Surface), the A:D ratio of Myc-GB1a is significantly higher than that of Myc-GB1b (mean $\pm$ SEM, *$p < 0.05$, Student’s $t$ test).
transfected neurons. Following transfection, neurons were fixed at DIV14, permeabilized, and stained with antibodies against the Myc-tag and the dendritic marker MAP2. We found that Myc-GB1a was present in axons, somata, and dendrites, whereas Myc-GB1b was restricted to the somatodendritic compartment (Fig. 2A). The A:D ratios of Myc-GB1a and Myc-GB1b were determined by normalizing the green Myc fluorescence intensity to the RFP fluorescence intensity in axons and dendrites (Gu et al., 2003; Sampo et al., 2003; Das and Banker, 2006). The A:D ratio for transfected Myc-GB1a was increased by 2.7-fold compared to transfected Myc-GB1b (Myc-GB1a: 0.38 ± 0.04, n = 10; Myc-GB1b: 0.14 ± 0.05, n = 10; p < 0.01) (Fig. 2B), analogous as with the endogenous \( \text{GABA}_B_{1a} \) and \( \text{GABA}_B_{1b} \) proteins in \( 1b^{-/-} \) and \( 1a^{-/-} \) neurons, respectively (Fig. 1D). This demonstrates that the trafficking of endogenous and transfected \( \text{GABA}_{B1} \) subunits is alike. Moreover, this indicates that neither putative compensatory mechanisms in the knock-out backgrounds nor the surrogate signal peptide interfere with trafficking. We nevertheless also determined the distribution patterns of \( \text{GABA}_{B1} \) proteins that are C-terminally tagged with the green fluorescent protein (GFP) and therefore contain their intrinsic signal peptides. The A:D ratio for GB1a-GFP was significantly increased by twofold compared to GB1b-GFP (GB1a-GFP: 0.49 ± 0.06, n = 7; GB1b-GFP: 0.25 ± 0.04, n = 7; p < 0.01), thus consolidating that the surrogate signal peptide and the intrinsic signal peptides lead to a comparable axonal versus dendritic distribution. Furthermore, we analyzed whether trafficking is influenced by the developmental stage of cultured neurons. In neurons at DIV21, the A:D ratio of Myc-GB1a was significantly increased compared to Myc-GB1b (Myc-GB1a: 0.49 ± 0.04, n = 6; Myc-GB1b: 0.25 ± 0.05, n = 6; p < 0.01) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), providing no evidence for a developmental regulation of trafficking.

The levels of Myc-GB1a and Myc-GB1b at the cell surface were too low for reliable quantification. Presumably, exogenous \( \text{GABA}_{B1} \) subunits compete with endogenous \( \text{GABA}_{B1} \) subunits for \( \text{GABA}_{B2} \), which is required for escorting \( \text{GABA}_{B1} \) to the plasma membrane (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). To increase surface expression levels of the exogenous \( \text{GABA}_{B1} \) proteins, we therefore coexpressed the \( \text{GABA}_{B2} \) protein with the individual Myc-GB1a and Myc-GB1b proteins. This allowed quantification of the Myc-fluorescence at the cell surface of nonpermeabilized cells. The Myc-fluorescence was normalized to the fluorescence of coexpressed RFP and the A:D ratio determined as described above. Surface Myc-GB1a exhibited a significantly increased A:D ratio compared to surface Myc-GB1b (Myc-GB1a: 0.50 ± 0.09, n = 10; Myc-GB1b: 0.26 ± 0.02, n = 10; p < 0.05) (Fig. 2B), demonstrating that \( \text{GABA}_{B1a} \) is also enriched over \( \text{GABA}_{B1b} \) at the axonal plasma membrane. In addition, comparison of the data in Figure 2B shows that significantly more \( \text{GABA}_{B1a} \) than \( \text{GABA}_{B1b} \) protein traffics to axons, regardless of whether or not exogenous \( \text{GABA}_{B2} \) is supplied to WT neurons. This demonstrates that the \( \text{GABA}_{B2} \) expression level does not

Figure 3. The SDs in \( \text{GABA}_{B2} \) mediate axonal localization. A, In Myc-GB1aCS, the disulfide bridges in the SDs, which are critical for ligand binding (Kirkitadze and Barlow, 2001), were disrupted by mutation of cysteines to serines. Myc-GB1a and Myc-GB1aCS were individually coexpressed with RFP in cultured hippocampal neurons. Neurons were fixed at DIV14, permeabilized, and stained with antibodies against the Myc-tag and the dendritic marker MAP2. We found that Myc-GB1a and Myc-GB1aCS were individually coexpressed with RFP in cultured hippocampal neurons. Neurons were fixed at DIV14, permeabilized, and stained with antibodies recognizing MAP2 (data not shown) and the Myc-tag. Merged images of the green Myc and the RFP fluorescence are shown at the top. Note that Myc-GB1aCS is excluded from axons. Scale bar, 10 μm. B, Myc-GB1aΔSD1 and Myc-GB1aΔSD2 proteins lacking either SD1 or SD2, respectively, both localize to axons and dendrites of transfected hippocampal neurons. Merged images of the green Myc and the RFP fluorescence are shown at the top. Scale bar, 10 μm. C, The A:D ratio of Myc-GB1aCS is significantly reduced compared to that of Myc-GB1a, while no significant reduction in the A:D ratios was observed for Myc-GB1aΔSD1 and Myc-GB1aΔSD2 (mean ± SEM, ***p < 0.001, 1-way ANOVA, Tukey’s post hoc test). D, Myc-GB1aCS and Myc-GB1a, when expressed together with GABA B2, activate Kir3.1/3.2 channels in transfected CHO cells to a similar extent. Calibration: 50 pA, 5 s.
markedly influence the axonal versus dendritic distribution of the GABA<sub>B1a</sub> and GABA<sub>B1b</sub> proteins.

GABA<sub>B2</sub> needs to coassemble with GABA<sub>B1a</sub> to traffic to the axonal compartment

We conversely investigated whether the subcellular localization of GABA<sub>B2</sub> is influenced by the GABA<sub>B1</sub> subunit isoforms. We analyzed the axonal versus dendritic distribution of transfected Myc-GB2 in WT as well as in 1<sup>a</sup>/<sup>-/-</sup> and 1<sup>b</sup>/<sup>-/-</sup> neurons. Myc-GB2 failed to efficiently traffic into axons in neurons of all genotypes, which is reflected by the similar A:D ratios (Myc-GB2 in WT: 0.31 ± 0.08, n = 10; Myc-GB2 in 1<sup>a</sup>/<sup>-/-</sup>: 0.32 ± 0.04, n = 10; Myc-GB2 in 1<sup>b</sup>/<sup>-/-</sup>: 0.29 ± 0.03, n = 10; p > 0.05). Presumably, the amount of endogenous GABA<sub>B1a</sub> protein is insufficient for efficient trafficking of Myc-GB2 into axons. Coexpression of exogenous Myc-GB1a but not Myc-GB1b significantly increased the A:D ratio of HA-GB2 in WT neurons (HA-GB2 + Myc-GB1a: 0.59 ± 0.10, n = 6; HA-GB2 + Myc-GB1b: 0.21 ± 0.05, n = 6; p < 0.01). This indicates that GABA<sub>B2</sub> is a somatodendritic protein that needs to coassemble with GABA<sub>B1a</sub> to reach the axonal compartment.

Each SD in GABA<sub>B1a</sub> can mediate axonal localization on its own

The SDs in GABA<sub>B1</sub> bind with low nanomolar affinity to binding sites in neuronal membranes (Tiao et al., 2008) and likely mediate axonal localization through interaction with other protein(s). To interact with binding partners the SDs in GABA<sub>B1a</sub> need to fold into a globular structure that is stabilized by disulfide bonds (Wei et al., 2001; Tiao et al., 2008). We therefore addressed whether the tertiary structure of the SDs is crucial for axonal localization of GABA<sub>B1a</sub>. In the Myc-GB1aCS mutant, we prevented disulfide bond formation in each of the SDs by converting two of the four conserved cysteines into serines. Following transfection into cultured hippocampal neurons, Myc-GB1aCS was robustly targeted to dendrites but not to axons (Fig. 3A). Accordingly, the A:D ratio in Myc-GB1aCS was significantly smaller than that for WT Myc-GB1a (Myc-GB1a: 0.41 ± 0.06, n = 8; Myc-GB1aCS: 0.14 ± 0.03, n = 10; p < 0.001) (Fig. 3C). Of note, the A:D ratio of Myc-GB1aCS was similar to that of Myc-GB1b (Fig. 2B). While Myc-GB1aCS failed to traffic to axons the mutant protein efficiently activated Kir3 channels when coexpressed with GABA<sub>B2</sub> (Fig. 3D). This demonstrates that interfering with the folding of the SDs impairs axonal trafficking without impairing receptor surface expression or G-protein signaling. Altogether, these results support that the SDs engage in interactions that are necessary for axonal localization of GABA<sub>B1a</sub>.

Structurally, the two SDs in GABA<sub>B1a</sub> differ from each other (Blein et al., 2004). The first SD shows conformational heterogeneity under a wide range of conditions and interacts with the extracellular matrix protein fibulin-2. The second SD is more compactly folded and exhibits strong structural similarity
with the SDs in proteins of the complement system. It is conceivable that the two SDs exert different functions and interact with different proteins. We therefore investigated whether each of the two SDs in GABA\(_{B1a}\) can mediate axonal targeting on its own. In the Myc-GB1a\(\Delta SD1\) and Myc-GB1a\(\Delta SD2\) mutants, we deleted either the first or the second SD, respectively (Fig. 3B). Myc-GB1a\(\Delta SD1\) and Myc-GB1a\(\Delta SD2\) were both efficiently targeted to axons, and the A:D ratios were not significantly different from that of WT Myc-GABAB1a (Myc-GB1a: 0.41 \pm 0.06, \(n = 8\); Myc-GB1a\(\Delta SD1\): 0.49 \pm 0.04, \(n = 9\); Myc-GB1a\(\Delta SD2\): 0.47 \pm 0.04, \(n = 8\); \(p > 0.05\)) (Fig. 3C). This shows that each of the two SDs in GABA\(_{B1a}\) can mediate axonal localization on its own.

The SDs of GABA\(_{B1a}\) polarize the uniformly distributed transmembrane protein CD8\(\alpha\) to axons

The SDs could promote axonal localization of GABA\(_{B1a}\) either by acting as axonal trafficking signals or, alternatively, by inactivating dendritic targeting signals, which would also result in a more uniform distribution. To distinguish between these two possibilities, we analyzed whether the SDs of GABA\(_{B1a}\) are capable of polarizing an unpolarized heterologous transmembrane protein, CD8\(\alpha\) (Jareb and Banker, 1998), to axons. We first confirmed that Myc-CD8\(\alpha\) uniformly distributes to axons and dendrites of transfected hippocampal neurons (Fig. 4). As expected for an unpolarized protein, the A:D ratio was with 1.24 \pm 0.07 (\(n = 24\)) close to 1. In contrast, when the two SDs of GABA\(_{B1a}\) were fused to the ectodomain of CD8\(\alpha\), the chimeric Myc-SDs-CD8\(\alpha\) protein clearly polarized to axons (A:D ratio 2.37 \pm 0.26, \(n = 24\); \(p < 0.001\) vs Myc-CD8\(\alpha\)) (Fig. 4). This clearly identifies the SDs as bona fide axonal targeting signals.

The SDs of GABA\(_{B1a}\) direct the somatodendritic mGluR1a protein to axons

According to our hypothesis, the SDs of GABA\(_{B1a}\) not only act as axonal trafficking signals but also override the dendritic targeting signals present in GABA\(_{B1a}\) and/or GABA\(_{B2}\). We therefore investigated whether the SDs of GABA\(_{B1a}\) can direct a somatodendritically localized heterologous transmembrane protein to axons. For this experiment, we used mGluR1a, a receptor with C-terminal dendritic trafficking signals (Francesconi and Duvoisin, 2002; Das and Banker, 2006). We confirmed that Myc-mGluR1a is highly expressed in the dendrites but excluded from the axons of transfected hippocampal neurons (Fig. 3A, B). When the two SDs of GABA\(_{B1a}\) were fused to the N-terminal ectodomain of mGluR1a, the chimeric Myc-SDs-mGluR1a protein readily trafficked to axons and exhibited a significantly higher A:D ratio than WT Myc-mGluR1a (Myc-mGluR1a: 0.03 \pm 0.06, \(n = 9\); Myc-SDs-mGluR1a: 1.26 \pm 0.15, \(n = 11\); \(p < 0.001\)). This shows that the SDs of GABA\(_{B1a}\) can override the somatodendritic targeting signals in the C terminus of mGluR1a.

**Surface expression is not required for axonal delivery of GABA\(_{B1a}\).**

GABA\(_{B1a}\) is not only present in the axons, but also highly expressed in the somatodendritic compartment (Figs. 1, 2). It is therefore conceivable that GABA\(_{B1a}\) reaches the axonal compartment through transcytosis from the somatodendritic compartment, similar to what is reported for the neuronal cell adhesion molecule NgCAM (Wisco et al., 2003). This dendrite-to-axon transcytotic pathway requires internalization of axonally bound proteins from the dendritic plasma membrane. We investigated whether Myc-GB1a can be transported into axons in the absence of surface expression. Since GABA\(_{B2}\) is necessary for surface localization of GABA\(_{B1}\) subunits (Margeta-Mitrovic et al., 2002; Pagano et al., 2001), we prevented surface trafficking of Myc-GB1a by expressing it in cultured hippocampal neurons of GABA\(_{B2}\)\(-/-\) (2\(-/-\)) mice (Gassmann et al., 2004). Myc-GB1a was transported into axons in the absence of GABA\(_{B2}\) (Fig. 6) and the A:D ratio in 2\(-/-\) neurons was not significantly different from that in WT neurons (Myc-GB1a in WT: 0.45 \pm 0.05, \(n = 12\); Myc-GB1a in 2\(-/-\): 0.40 \pm 0.04, \(n = 19\); \(p > 0.05\)). This corroborates that Myc-GB1a reaches the axonal compartment via an intracellular route, independent of any surface expression. Lateral diffusion of surface receptors is therefore not necessary for axonal localization of GABA\(_{B1a}\). However, the SDs are not only involved in axonal delivery of GABA\(_{B}\) receptors but also in their retention at the cell surface of the terminal (Tiao et al., 2008). Lateral diffusion and selective retention could therefore, in principle, contribute to the pool of axonal GABA\(_{B1a}\) receptors. It was recently proposed that proteins not only traffic into axons via post-Golgi transport vesicles but also within the endoplasmic reticulum (ER), from where proteins are released via exit sites (Aridor and Fish, 2009; Merianda et al., 2009). It is therefore conceivable that GABA\(_{B1a}\) traffics into axons within the ER. As previously reported (Ramirez et al., 2009), we found a partial colocalization of transfected GABA\(_{B1a}\) subunits with the ER in the somatodendritic compartment using an ER-targeted GFP (Aoki et al., 2002) as a marker (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). We also observed a partial colocalization of transfected GABA\(_{B1a}\) with ER-targeted GFP in axons, making it conceivable that some GABA\(_{B1a}\) also enters axonal ER. However, according to prevailing concepts axonally destined proteins traffic in intracellular post-Golgi transport vesicles to the terminals (Horton and Ehlers, 2003). We therefore expect that intracellular GABA\(_{B1a}\) in axons is mostly present in transport vesicles delivering their cargo to the terminal.
Discussion
The SDs of GABA\textsubscript{B1a} are axonal targeting signals
We previously reported that selectively the GABA\textsubscript{B1a} protein traffics into the axons of pyramidal neurons in organotypic slice cultures, while both the GABA\textsubscript{B1a} and GABA\textsubscript{B1b} proteins traffic to dendrites (Vigot et al., 2006). The reason for this difference in axonal trafficking is not obvious. GABA\textsubscript{B1a} only differs from GABA\textsubscript{B1b} by the presence of a pair of SDs at its N terminus. A classical scenario whereby GABA\textsubscript{B1b} traffics to the dendrites by unique C-terminal dendritic targeting signal(s) and GABA\textsubscript{B1a} distributes more uniformly due to the absence of such signal(s) is therefore ruled out. A plausible hypothesis is that GABA\textsubscript{B1a} and GABA\textsubscript{B1b} are retained in the somatodendritic compartment by common dendritic targeting signal(s) in GABA\textsubscript{B1a} and/or the associated GABA\textsubscript{B2} subunit. A fraction of GABA\textsubscript{B1a} protein would then be directed to axons by dominant axonal targeting signal(s) or signals that inactivate the dendritic signal(s), which would also result in a more randomized distribution. We now report that the SDs in GABA\textsubscript{B1a} function as bona fide axonal targeting signals. When fused to the extracellular/luminal domain of CD8ε the SDs efficiently polarize this prototypical unpolarized protein (Jareb and Banker, 1998) to axons. Likewise, when fused to mGluR1α the SDs direct this somatodendritic protein to axons, directly showing that the SDs can override C-terminal dendritic targeting signals (Francesconi and Duvoisin, 2002; Das and Banker, 2006). SDs are also present in other neuronal proteins, for example in the “CUB and sushi multiple domains 1” (CSMD1) and Sez-6 proteins. CSMD1 is a membrane component of the distal tip of growing axons (Kraus et al., 2006). It remains an interesting possibility that SDs mediate the axonal localization of this protein. The Sez-6 protein isoforms are predominantly expressed in the somatodendritic compartment but also present at the axon terminal (Gunnersen et al., 2007). Trafficking of Sez-6 proteins to axons could therefore also depend on the SDs and involve a mechanism that overrules dendritic signals, in the same way as now proposed for GABA\textsubscript{B1a}.

We show that the tertiary structure of the SDs is critical for axonal localization of GABA\textsubscript{B1a}. Since the SDs of GABA\textsubscript{B1a} recognize binding sites in neuronal membranes (Tiao et al., 2008), they probably engage in interactions that direct axonal localization. Our observation that each of the two SDs mediates axonal localization on its own suggests that they interact with proteins of similar function or with binding sites within the same protein. The SDs confer axonal localization in the absence of GABA\textsubscript{B1a} surface expression, suggesting that they bind to axonally destined proteins in the lumen of the trans-Golgi network (TGN). Such a mechanism for axonal targeting has been suggested for NgCAM, which uses five fibronectin type-III like repeats in its ectodomain as targeting signals (Sampo et al., 2003). It was recently proposed that the elements of a mature presynaptic terminal, e.g., calcium channel subunits, endocytic proteins and synaptic vesicle proteins are transported along axons as discrete “transport packets” (Ahmari et al., 2000). Since GABA\textsubscript{B1} receptors are localized near the active zone (Kulik et al., 2003) it is plausible that GABA\textsubscript{B1a} is transported “piggyback style” by interacting with presynaptic proteins in the lumen of transport vesicles, similar to other axonally destined proteins (Roos and Kelly, 2000). Since GABA\textsubscript{B1a} partially colocalizes with ER-targeted GFP in the axons, it is possible that some GABA\textsubscript{B1a} protein also reaches the axon within the ER (Aridor and Fish, 2009; Merianda et al., 2009). This would imply the existence of a SD-dependent mechanism that selectively distributes GABA\textsubscript{B1a} but not GABA\textsubscript{B1b} to the axonal ER. It is interesting to note that functionally relevant binding sites for the SDs in GABA\textsubscript{B1a} also exist at the cell surface of glutamatergic terminals (Tiao et al., 2008). It remains to be seen whether these extracellular binding sites are identical with the intracellular binding sites regulating axonal trafficking.

Our model for the differential targeting of GABA\textsubscript{B1} isoforms proposes the existence of dendritic targeting signals in the GABA\textsubscript{B1} and/or GABA\textsubscript{B2} subunits. Dendritic targeting signals in transmembrane proteins are generally confined to cytoplasmic domains (West et al., 1997; Jareb and Banker, 1998; Poyatos et al., 2000; Rivera et al., 2003; Hirokawa and Takemura, 2005). Both the C-terminal domain of the GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits contain a number of putative dendritic targeting signals. It was recently proposed that GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits are transported into dendrites while still residing in the ER and before assembly into heteromeric complexes (Vidal et al., 2007; Ramirez et al., 2009). Consistent with this proposal, we found a colocalization of transfected GABA\textsubscript{B1} protein with ER-targeted GFP. Possibly, GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits do not require dendritic targeting signals in their primary sequence if transported to dendrites within the ER.

Conditional activation of axonal and somatodendritic targeting signals can explain GABA\textsubscript{A} receptor distribution
Our observation that GABA\textsubscript{B1a} is transported into axons without preceding cell surface expression rules out selective retention at the plasma membrane and dendrite-to-axon transcytosis as the mechanism for axonal localization (Wisco et al., 2003). Overall, our findings are most compatible with the “selective delivery” model for axonal trafficking (Horton and Ehlers, 2003; Sampo et al., 2003; Wisco et al., 2003). In this model both the GABA\textsubscript{B1a} and GABA\textsubscript{B1b} subunits are transported into dendrites in somatodendritic post-Golgi carriers. Additionally, some GABA\textsubscript{B1b} subunits are transported to axons in distinct axonal carriers. Somatodendritic targeting signals, residing within the C-terminal domain of GABA\textsubscript{B1} and/or GABA\textsubscript{B2}, would sort GABA\textsubscript{B1a} to the default somatodendritic compartment unless the SDs bind to axonally destined protein(s) in the lumen of the TGN. The availability of this putative SD-binding protein(s) would represent a limiting factor for sorting of GABA\textsubscript{B1a} into axonal transport carriers and explain why much of the GABA\textsubscript{B1a} protein resides in the somatodendritic compartment. A prerequisite for the “selective delivery” model is that the luminal SDs can silence dendritic targeting signal(s) in GABA\textsubscript{B1a} and/or GABA\textsubscript{B2} on the opposite side of the membrane. Our experiments with mGluR1α directly show that luminal SDs can inactivate somatodendritic targeting signals across the membrane, suggesting that they function similarly in the structurally related GABA\textsubscript{B1} receptors. Of note, conformational changes in the extracellular domain of GABA\textsubscript{B1} are allosterically coupled to conformational changes in the intracellular domains of GABA\textsubscript{B1} and GABA\textsubscript{B2} (Parmentier et al., 2002). This could explain how binding to the SDs leads to the unbinding of dendritic sorting adaptors across the membrane. Of physiological relevance, the conditional activation of axonal trafficking signals may provide a means to adjust the strength of presynaptic GABAergic inhibition. Finally, on a different note, the SDs of GABA\textsubscript{B1a} are a potentially useful experimental tool for delivering transmembrane proteins to axons.

References
Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. Nat Neurosci 3:445–451.
Alle H, Geiger JR (2006) Combined analog and action potential coding in hippocampal mossy fibers. Science 311:1290–1293.
Ango F, Albani-Torregrossa S, Joly C, Robbe D, Michel JM, Pin JP, Bockeart J, Fagni L (1999) A simple method to transfer plasmid DNA into neuronal primary cultures: functional expression of the mGlu5 receptor in cerebellar granule cells. Neuropharmacology 38:793–803.

Aoki S, Su Q, Li H, Nishikawa K, Ayukawa K, Hara Y, Namikawa K, Kiryu-Seo S, Kiyama H, Wada K (2002) Identification of an axotomy-induced glycosylated protein, AIGP1, possibly involved in cell death triggered by endoplasmic reticulum–Golgi stress. J Neurosci 22:10751–10760.

Aridor M, Fish KN (2009) Selective targeting of ER exit sites supports axon development. Traffic 10:1669–1684.

Benson DL, Watkins FH, Steward O, Banker G (1994) Characterization of GABAergic neurons in hippocampal cell cultures. J Neurocytol 23:279–295.

Blein S, Ginhin R, Uhrin D, Smith BO, Soares DC, Veltel S, McIlhinney RA, Benson DL, Watkins FH, Steward O, Banker G (1994) Characterization of GABAergic neurons in hippocampal cell cultures. J Neurocytol 23:279–295.

Bowler S, Bouguet N, Seddik R, Vigot R, Turecek R, Gassmann M, Vogt KE, Braeuner-Kuegler S, Jan YN, Jan LY (2003) A conserved domain in axonal targeting of Kv1 Shaker voltage-gated potassium channels. Science 301:646–649.

Bose SM, Meloni BP, Arthur PG, Bojarski C, Knuckey NW (2006) Improved superfusion technique for rapid cooling or heating of cortical neuronal cultures. Brain Res 1102:27–38.

Bower NG, Betterl B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002) International union of pharmacology. XXXIII. Mammalian γ-aminobutyric acidA receptors: structure and function. Pharmacol Rev 54:247–264.

Breuer GJ, Torricelli JR, Egee EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35:567–576.

Caceres A, Binder LF, Payne MR, Bender P, Rehlin H, L水肿ard O (1984) Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. J Neurosci 4:394–410.

Couvée A, Moss SJ, Pangalos MN (2000) GABAB receptors: a new paradigm with monoclonal hybridoma antibodies. Mol Cell Neurosci 15:178–185.

Davies PJ, Petrou S, Faber ES, Sah P, Tan SS (2007) Sez-6 proteins affect dendritic spine morphology and expression of a metabotropic glutamate receptor. Nature 441:390–430.

Diettert I, Benedikt J, Vyklicky L, Zimmermann K, Reeh PW, Vlachova V (2006) Improved superfusion technique for rapid cooling or heating of cultured cells under patch-clamp conditions. J Neurosci Methods 151:178–185.

Dounce EK, Voros T, Shi QL, Norman D, Kirkedal M, Barlow PN (1998) Demonstration of a tandem pair of complement protein modules in GABAB receptor 1a. FEBS Lett 432:103–108.
(2009) Dendritic assembly of heteromeric gamma-aminobutyric acid type B receptor subunits in hippocampal neurons. J Biol Chem 284:13077–13085.

Rivera JF, Ahmad S, Quick MW, Liman ER, Arnold DB (2003) An evolutionarily conserved dileucine motif in Shal K⁺ channels mediates dendritic targeting. Nat Neurosci 6:243–250.

Roos J, Kelly RB (2000) Preassembly and transport of nerve terminals: a new concept of axonal transport. Nat Neurosci 3:415–417.

Sampo B, Kaech S, Kunz S, Banker G (2003) Two distinct mechanisms target membrane proteins to the axonal surface. Neuron 37:611–624.

Schuler V, Lüscher C, Blanchet C, Klix N, Sansig G, Klehs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigoureux JM, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaumann K, van der Putten H, Bettler B (2001) Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABAergic responses in mice lacking GABAβ1. Neuron 31:47–58.

Shaban H, Humeau Y, Henry C, Cassasus G, Shigemoto R, Ciocchi S, Barbieri S, van der Putten H, Kaumann K, Bettler B, Lüthi A (2006) Generalization of amygdala LTP and conditioned fear in the absence of presynaptic inhibition. Nat Neurosci 9:1028–1035.

Soares DC, Barlow PN (2005) Complement control protein modules in the regulators of complement activation. In: Structural biology of the complement system (Morikis D, Lambris JD, eds), pp 19–62. Boca Raton, FL: CRC, Taylor and Francis Group.

Steiger JL, Bandyopadhyay S, Farb DH, Russe SJ (2004) cAMP response element-binding protein, activating transcription factor-4, and upstream stimulatory factor differentially control hippocampal GABAergic and GABABergic subunit gene expression through alternative promoters. J Neurosci 24:6115–6126.

Tiao JY, Bradaia A, Biermann B, Kaufmann K, Metz M, Haller C, Rolink AG, Pless E, Barlow PN, Gassmann M, Bettler B (2008) The sushi domains of secreted GABAergic isoforms selectively impair GABAergic heteroreceptor function. J Biol Chem 283:31005–31011.

Ulrich D, Bettler B (2007) GABAergic receptor: synaptic functions and mechanisms of diversity. Curr Opin Neurobiol 17:298–303.

Ulrich D, Besseyrias V, Bettler B (2007) Functional mapping of GABAergic receptor subtypes in the thalamus. J Neurophysiol 98:3791–3795.

Vidal RL, Ramírez OA, Sandoval L, Koenig-Robert R, Härtel S, Couve A (2007) Marlin-1 and conventional kinesin link GABAergic receptors to the cytoskeleton and regulate receptor transport. Mol Cell Neurosci 35:501–512.

Vigot R, Barbieri S, Bräuner-Osborne H, Turecek R, Shigemoto R, Zhang YP, Luján R, Jacobson LH, Biermann B, Fritschy JM, Vacher CM, Muller M, Sansig G, Guett N, Cryan JF, Kaufmann K, Gassmann M, Oertner TG, Bettler B (2006) Differential compartmentalization and distinct functions of GABAergic receptor variants. Neuron 50:589–601.

Wei X, Orchardson M, Gracie JA, Leung BP, Gao B, Guan H, Niedbala W, Paterson GK, McInnes IB, Liew FY (2001) The Sushi domain of soluble IL-15 receptor alpha is essential for binding IL-15 and inhibiting inflammatory and allogenic responses in vitro and in vivo. J Immunol 167:277–282.

West AE, Neve RL, Buckley KM (1997) Identification of a somatodendritic targeting signal in the cytoplasmic domain of the transferrin receptor. J Neurosci 17:6038–6047.

Winckler B, Mellman I (1999) Neuronal polarity: controlling the sorting and diffusion of membrane components. Neuron 23:637–640.

Wisco D, Anderson ED, Chang MC, Norden C, Boiko T, Folsch H, Winckler B (2003) Uncovering multiple axonal targeting pathways in hippocampal neurons. J Cell Biol 162:1317–1328.

Yamada J, Saitow F, Satake S, Kiyohara T, Konishi S (1999) GABAergic receptor-mediated presynaptic inhibition of glutamatergic and GABAergic transmission in the basolateral amygdala. Neuropharmacology 38:1743–1753.