Cellular/Molecular

The Sushi Domains of $GABA_B$ Receptors Function as Axonal Targeting Signals

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$GABA_B$ receptors are the G-protein-coupled receptors for GABA, the main inhibitory neurotransmitter in the brain. Two receptor subtypes, $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$, are formed by the assembly of $GABA_{B1a}$ and $GABA_{B1b}$ subunits with $GABA_{B2}$ subunits. The $GABA_{B1b}$ 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 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 $GABA_{B(1a,2)}$ 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 other neurotransmitters (heteroreceptors), while postsynaptic $GABA_B$ 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, $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ (Marshall et al., 1999). Most if not all neurons in the CNS coexpress $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ 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-Garci 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 $GABA_{B(1a,2)}$ receptors inhibit glutamate release in response to endogenous GABA, while both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors mediate postsynaptic inhibition. This is a consequence of a selective trafficking of $GABA_{B(1a,2)}$ 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 $GABA_{B1a}$

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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 $GABA_{B(1b,2)}$ receptor is the somatodendritic compartment, this suggests that the longer $GABA_{B(1a,2)}$ receptor also contains common dendritic targeting signals in either the $GABA_{B1a}$ or the associated $GABA_{B2}$ subunit. This implies a mechanism that prevents a fraction of $GABA_{B(1a,2)}$ receptors from trafficking to the default somatodendritic compartment and instead directs them to axons.

Here, we report that $GABA_{B(1a,2)}$ 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; Vigot 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 (Pagano 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 MVLLLILSVLLLKEDVRGSAQS, followed by the Myc-tag, TREQKLISEEDLTR [replaced residues: Myc-GB1a, 1-16 (Kaupmann et al., 1997); Myc-GB1b, 1-29 (Kaupmann et al., 1997); Myc-mGluR1a, 1-20 (Masu et al., 1991); Myc-CD8α, 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 29, 95, 99, and 156 (Kaupmann et al., 1997) were mutated to serine residues by site-directed mutagenesis of thymine to adenine. To generate Myc-GB1aΔSD1 and Myc-GB1aΔSD2, residues G^{28} to C^{95} or V^{96} to Q^{157} of Myc-GB1a were deleted. To generate Myc-SDs-mGluR1a, residues G^{17} to S^{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-CD8α, the residues G^{17} to S^{134} of $GABA_{B1a}$ were introduced after the Myc-tag in Myc-CD8α (CD8α 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 pMH4-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 M borate buffer (boric acid/sodium tetraborate). Neurons were seeded at low density (~100–150 cells/mm^2) for endogenous $GABA_{B1}$ labeling or at high density (~750 cells/mm^2) for transfection experiments or electrophysiological recordings and then incubated at 37°C/5% CO_2. Low-density cultures were cultivated in HC-MEM medium [1× MEM with Glutamax, 0.3% glucose (w/v), 10% horse serum, and 1% 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 [1× 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 50–100 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 (pMH4-SYN-tdimer2-RFP; 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 CGP54626 (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 $GABA_B$ 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 gluconate, 32.5 KCl, 10 HEPES, 5 EGTA, 4 MgATP, 0.6 NaGTP, 10 Tris phosphocreatine; pH 7.2, 297 mOsm. $GABA_B$ responses were evoked by application of baclofen (10 s) (Dittert 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_2HPO_4, 1.5 mM KH_2PO_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 1× 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-$GABA_{B1}$-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 (1:500; 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.

Microscopy. Immunolabeled neurons were viewed at room temperature 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; Obermair 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 filters used to detect secondary antibodies were as follows: L5-filter for Alexa goat anti-mouse 488 (Myc antibody), Y5-filter for Alexa goat anti-chicken 647 (MAP2 antibody), Y3-filter for RFP or Alexa goat anti-rabbit 568 (polyclonal $GABA_{B1}$ antibody). The ER-targeted GFP was

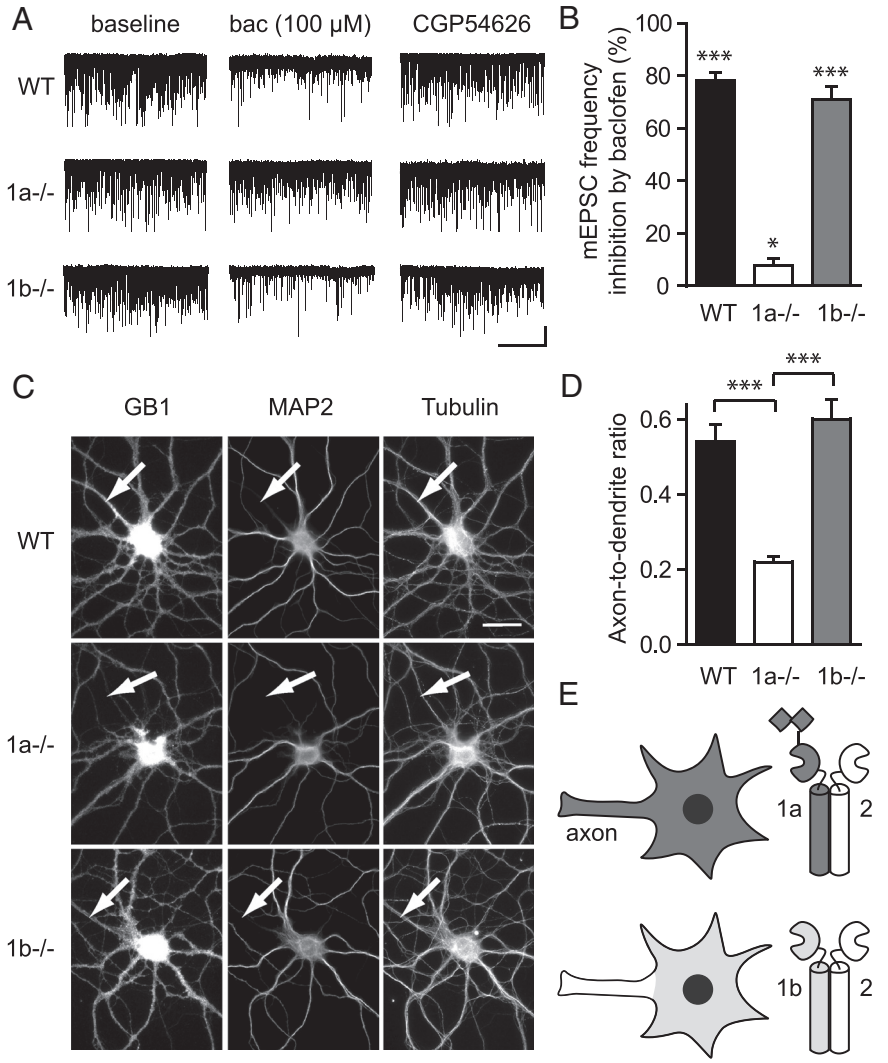

Figure 1. Endogenous $GABA_{B(1a,2)}$ but not $GABA_{B(1b,2)}$ receptors are present in axons and inhibit glutamate release in cultured hippocampal neurons. ***A***, Representative mEPSC recordings under baseline conditions, during baclofen (100 μM) application and after antagonizing $GABA_B$ receptors with CGP54626 (1 μM) in WT, $1a^{-/-}$, and $1b^{-/-}$ neurons. Calibration: 20 pA, 25 s. ***B***, Summary bar graph illustrating that baclofen strongly inhibits the frequency of mEPSCs in WT (78.1 ± 3.1%, $n = 16$) and $1b^{-/-}$ (70.8 ± 5.1%, $n = 15$) neurons, but not in $1a^{-/-}$ (7.7 ± 2.8%, $n = 10$) neurons. Values are means ± SEM, one-sample *t* test, $^*p < 0.05$, $^{***}p < 0.001$. ***C***, Cultured hippocampal neurons from WT, $1a^{-/-}$, and $1b^{-/-}$ mice were fixed, permeabilized, and stained with antibodies recognizing $GABA_{B1a}$ and $GABA_{B1b}$ (GB1), the dendritic marker protein MAP2, or the cytoskeleton protein tubulin. Arrows mark MAP2-negative axons. Note the lack of GB1 immunolabeling in axons of $1a^{-/-}$ neurons. Scale bar, 50 μm. ***D***, A:D ratio of the endogenous $GABA_{B1}$ proteins in WT, $1a^{-/-}$, and $1b^{-/-}$ neurons. The fluorescence intensity of GB1 immunolabeling was normalized to the fluorescence intensity of tubulin immunolabeling. The A:D ratio of $GABA_{B1}$ protein is significantly smaller in $1a^{-/-}$ compared to WT and $1b^{-/-}$ neurons (mean ± SEM, $^{***}p < 0.001$, 1-way ANOVA with Tukey's *post hoc* test). ***E***, Schematic depiction of endogenous $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptor distribution in cultured hippocampal neurons and hippocampal slice culture. Squares indicate the two in tandem arranged SDs at the N terminus of $GABA_{B1a}$.

from Clontech. Pictures were taken with each filter separately. Pictures from the endogenous $GABA_{B1}$ staining were captured using immersion oil without autofluorescence (Leica Microsystems catalog #11513859) and a 63× oil objective with 1.32 NA (HCX PL APO). Images to evaluate the axonal versus dendritic distribution of heterologously expressed $GABA_B$ 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 $GABA_{B1}$ 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 with $GABA_{B1}$ immunostaining. Average pixel intensities were determined along the traced lines and in the background rectangles. After background subtraction, the anti-$GABA_{B1}$ fluorescence intensity was normalized to the anti-tubulin fluorescence intensity in axon and dendrites. The normalized data were used to determine the A:D ratio. The A:D ratio of Myc-tagged constructs was determined by normalizing the Myc-labeling to the RFP labeling (Gu et al., 2003; Sampo et al., 2003). Cells to be analyzed were selected using the soluble RFP fill and only considered for quantification if the RFP fluorescence was evenly distributed over the entire neuron, including distal axons and dendrites. Cells expressing constructs at very high levels were excluded from analysis because such cells exhibit a less polarized distribution of expressed proteins. Seven to nineteen neurons from at least two independent culture preparations for each construct were analyzed. SPSS or GraphPad PRISM software was used for statistical analysis.

Reagents. TTX was from Latoxan. Baclofen and CGP54626 were from Novartis Pharma. HEPES was from AppliChem (catalog #A1069.0100). All other reagents were from Fluka/Sigma.

Results

Endogenous $GABA_{B1a}$ but not $GABA_{B1b}$ 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 $GABA_{B1a}$ and $GABA_{B1b}$ subunits in glutamatergic neurons. We first investigated whether cultured pyramidal neurons preserve the selective association of $GABA_{B(1a,2)}$ receptors with glutamatergic terminals seen in hippocampal slices (Vigot et al., 2006; Guetg et al., 2009). Specifically, we addressed whether functional $GABA_B$ heteroreceptors are present in cultured pyramidal neurons of $1b^{-/-}$ mice, but absent in neurons of $1a^{-/-}$ mice. Activation of $GABA_B$ heteroreceptors by baclofen, a $GABA_B$ 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. 1*A*,*B*). This confirms that functional $GABA_B$ 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 $GABA_{B(1b,2)}$ receptors are present at glutamatergic terminals. Alternatively, baclofen

may also activate somatic $GABA_{B(1b,2)}$ 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. 1*C*). 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 $GABA_{B1}$ protein is localized somatodendritically (WT: 0.54 ± 0.05, $n = 7$; $1a^{-/-}$: 0.22 ± 0.02, $n = 7$; $1b^{-/-}$: 0.60 ± 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. 1*D*), 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. 1*E*).

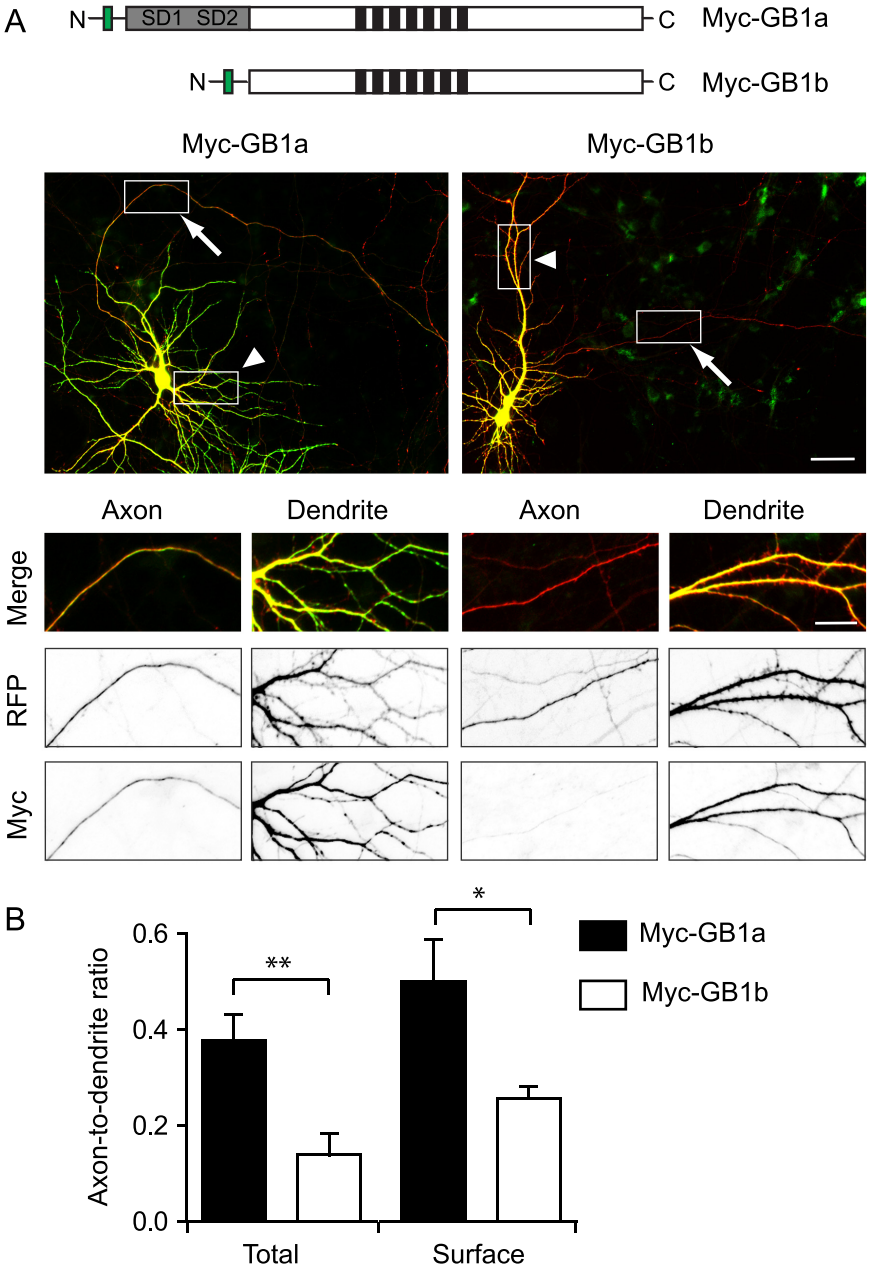

Figure 2. Exogenous $GABA_{B1a}$ but not $GABA_{B1b}$ protein localizes to the axons of transfected hippocampal neurons in culture. ***A***, Scheme of the tagged $GABA_{B1}$ isoforms (top). The gray bar indicates the two SDs (SD1, SD2) in $GABA_{B1a}$, the green bar the Myc-tag, and black bars the 7 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 μm; bottom, 10 μm. ***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).

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 (Ango 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

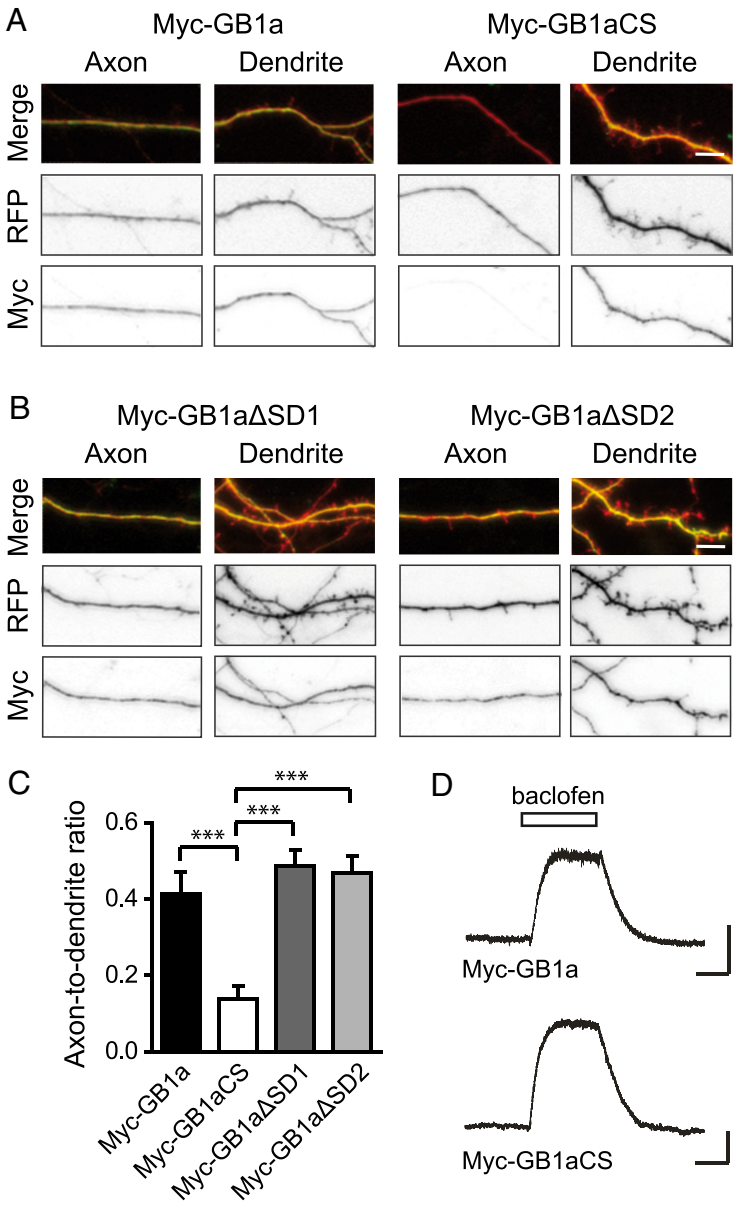

Figure 3. The SDs in $GABA_{B1a}$ mediate axonal localization. ***A***, In Myc-GB1aCS, the disulfide bridges in the SDs, which are critical for ligand binding (Kirkitadze and Barlow, 2001), were disrupted by mutation of cysteines to serines. Myc-GB1a and Myc-GB1aCS were individually coexpressed with RFP in cultured hippocampal neurons. Neurons were fixed at DIV14, permeabilized, and stained with antibodies 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.

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. 2*A*). 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 Myc-GB1b (Myc-GB1a: 0.38 ± 0.04, $n = 10$; Myc-GB1b: 0.14 ± 0.05, $n = 10$; $p < 0.01$) (Fig. 2*B*), analogous as with the endogenous $GABA_{B1a}$ and $GABA_{B1b}$ proteins in $1b^{-/-}$ and $1a^{-/-}$ neurons, respectively (Fig. 1*D*). This demonstrates that the trafficking of endogenous and transfected $GABA_{B1}$ 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 $GABA_{B1}$ 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 $GABA_{B1}$ subunits compete with endogenous $GABA_{B1}$ subunits for $GABA_{B2}$, which is required for escorting $GABA_{B1}$ to the plasma membrane (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). To increase surface expression levels of the exogenous $GABA_{B1}$ proteins, we therefore coexpressed the $GABA_{B2}$ 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. 2*B*), demonstrating that $GABA_{B1a}$ is also enriched over $GABA_{B1b}$ at the axonal plasma membrane. In addition, comparison of the data in Figure 2*B* shows that significantly more $GABA_{B1a}$ than $GABA_{B1b}$ protein traffics to axons, regardless of whether or not exogenous $GABA_{B2}$ is supplied to WT neurons. This demonstrates that the $GABA_{B2}$ expression level does not

markedly influence the axonal versus dendritic distribution of the $GABA_{B1a}$ and $GABA_{B1b}$ proteins.

$GABA_{B2}$ needs to coassemble with $GABA_{B1a}$ to traffic to the axonal compartment

We conversely investigated whether the subcellular localization of $GABA_{B2}$ is influenced by the $GABA_{B1}$ subunit isoforms. We analyzed the axonal versus dendritic distribution of transfected Myc-GB2 in WT as well as in $1a^{-/-}$ and $1b^{-/-}$ 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 $1a^{-/-}$: 0.32 ± 0.04, $n = 10$; Myc-GB2 in $1b^{-/-}$: 0.29 ± 0.03, $n = 10$; $p > 0.05$). Presumably, the amount of endogenous $GABA_{B1a}$ 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 $GABA_{B2}$ is a somatodendritic protein that needs to coassemble with $GABA_{B1a}$ to reach the axonal compartment.

Each SD in $GABA_{B1a}$ can mediate axonal localization on its own

The SDs in $GABA_{B1a}$ 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 $GABA_{B1a}$ 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 $GABA_{B1a}$. 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. 3*A*). 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. 3*C*). Of note, the A:D ratio of Myc-GB1aCS was similar to that of Myc-GB1b (Fig. 2*B*). While Myc-GB1aCS failed to traffic to axons the mutant protein efficiently activated Kir3 channels when coexpressed with $GABA_{B2}$ (Fig. 3*D*). 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 $GABA_{B1a}$.

Structurally, the two SDs in $GABA_{B1a}$ 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

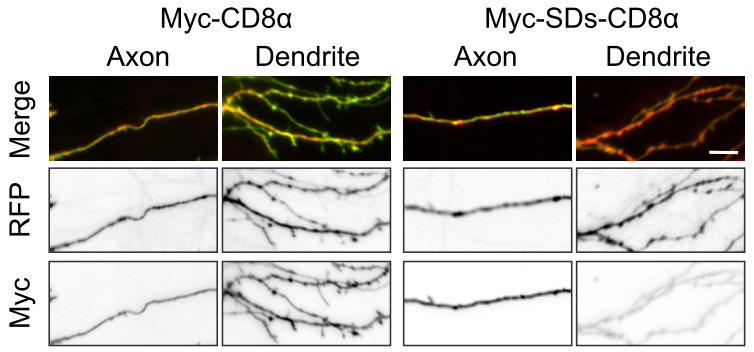

Figure 4. The SDs of $GABA_{B1a}$ function as axonal targeting signals in the heterologous CD8α protein. In Myc-SDs-CD8α, the SDs of $GABA_{B1a}$ were fused to the extracellular N-terminal domain of CD8α. Myc-CD8α or Myc-SDs-CD8α were individually coexpressed with RFP in cultured hippocampal neurons. Neurons were fixed at DIV14, permeabilized, and stained with antibodies recognizing MAP2 (data not shown) or the Myc-tag. Merged images of green Myc and RFP fluorescence are shown on top. Note that Myc-SDs-CD8α is barely detectable in dendrites, but highly expressed in axons. Scale bar, 10 μm.

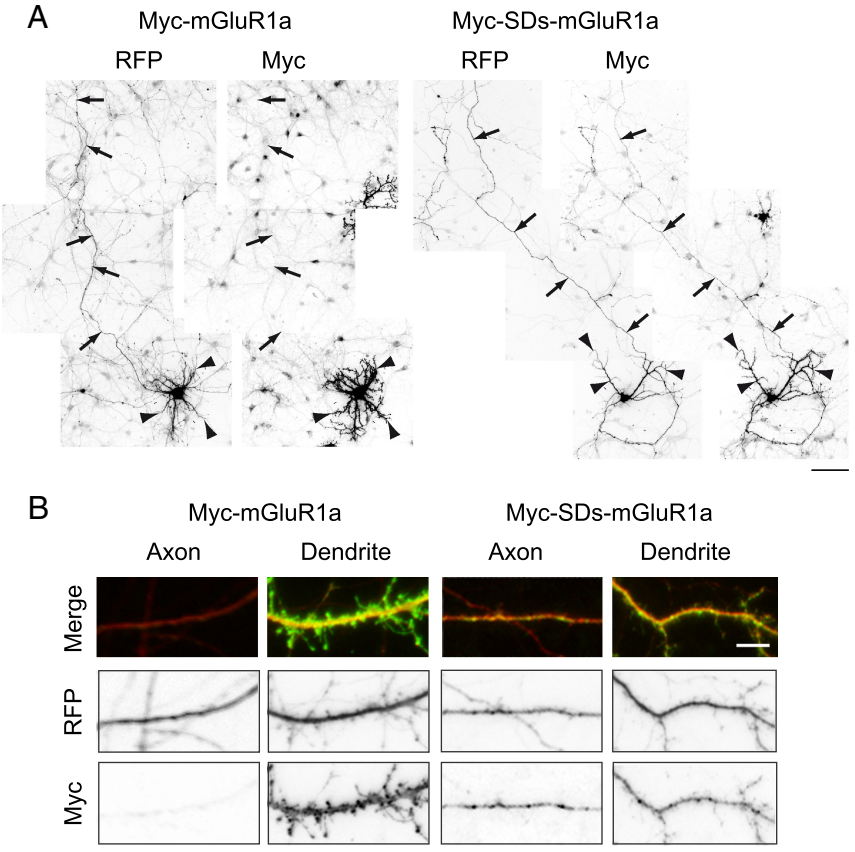

Figure 5. The SDs of $GABA_{B1a}$ redirect the somatodendritic mGluR1a protein to axons. ***A***, In Myc-SDs-mGluR1a, the two SDs of $GABA_{B1a}$ were fused to the extracellular N-terminal domain of mGluR1a. Myc-mGluR1a and Myc-SDs-mGluR1a were individually coexpressed with RFP in cultured hippocampal neurons. Neurons were fixed at DIV14, permeabilized, and stained with an antibody recognizing the Myc-tag. Arrows indicate the axon, arrowheads the dendrites. Note that Myc-SDs-mGluR1a but not Myc-mGluR1a is expressed in the axon. Scale bar, 25 μm. ***B***, Sections of axons and dendrites of neurons expressing Myc-mGluR1a and Myc-SDs-mGluR1a. Merged images of green Myc and RFP fluorescence are shown on top. Scale bar, 10 μm.

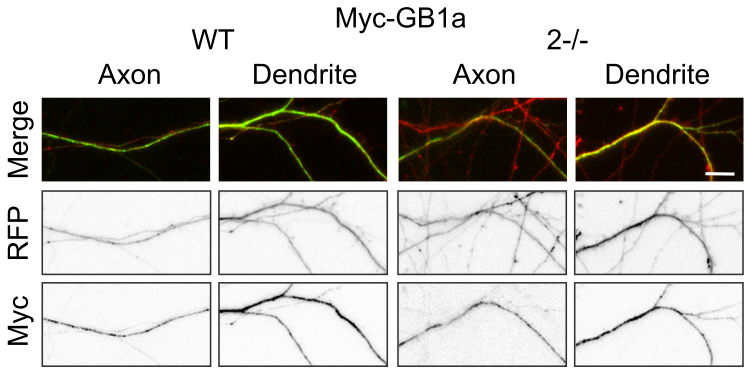

Figure 6. Surface expression is not required for axonal trafficking of $GABA_{B1a}$. Axonal and dendritic sections of cultured WT or $GABA_{B2}{}^{-/-}$ ($2^{-/-}$) hippocampal neurons expressing Myc-GB1a and RFP. Neurons were fixed at DIV14, permeabilized, and stained with antibodies recognizing MAP2 (data not shown) or the Myc-tag. Merged images of green Myc and RFP fluorescence are shown on top. Scale bar, 10 μm.

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-GB1aΔSD1 and Myc-GB1aΔSD2 mutants, we deleted either the first or the second SD, respectively (Fig. 3*B*). Myc-GB1aΔSD1 and Myc-GB1aΔSD2 were both efficiently targeted to axons, and the A:D ratios were not significantly different from that of WT Myc-GB1a (Myc-GB1a: 0.41 ± 0.06, $n = 8$; Myc-GB1aΔSD1: 0.49 ± 0.04, $n = 9$; Myc-GB1aΔSD2: 0.47 ± 0.04, $n = 8$; $p > 0.05$) (Fig. 3*C*). 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 CD8α 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, CD8α (Jareb and Banker, 1998), to axons. We first confirmed that Myc-CD8α 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 ± 0.07 ($n = 24$) close to 1. In contrast, when the two SDs of $GABA_{B1a}$ were fused to the ectodomain of CD8α, the chimeric Myc-SDs-CD8α protein clearly polarized to axons (A:D ratio 2.37 ± 0.26, $n = 24$; $p <$ 0.001 vs Myc-CD8α) (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. 5*A*,*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 ± 0.06, $n = 9$; Myc-SDs-mGluR1a: 1.26 ± 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., 2000; 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 ± 0.05, $n = 12$; Myc-GB1a in $2^{-/-}$: 0.40 ± 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 (Ramírez 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 $GABA_{B1a}$ are axonal targeting signals

We previously reported that selectively the $GABA_{B1a}$ protein traffics into the axons of pyramidal neurons in organotypic slice cultures, while both the $GABA_{B1a}$ and $GABA_{B1b}$ proteins traffic into dendrites (Vigot et al., 2006). The reason for this difference in axonal trafficking is not obvious. $GABA_{B1a}$ only differs from $GABA_{B1b}$ by the presence of a pair of SDs at its N terminus. A classical scenario whereby $GABA_{B1b}$ traffics to the dendrites by unique C-terminal dendritic targeting signal(s) and $GABA_{B1a}$ distributes more uniformly due to the absence of such signal(s) is therefore ruled out. A plausible hypothesis is that $GABA_{B1a}$ and $GABA_{B1b}$ are retained in the somatodendritic compartment by common dendritic targeting signal(s) in $GABA_{B1}$ and/or the associated $GABA_{B2}$ subunit. A fraction of $GABA_{B1a}$ 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 $GABA_{B1a}$ function as bona fide axonal targeting signals. When fused to the extracellular/luminal domain of CD8α the SDs efficiently polarize this prototypical unpolarized protein (Jareb and Banker, 1998) to axons. Likewise, when fused to mGluR1a 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 overrides dendritic signals, in the same way as now proposed for $GABA_{B1a}$.

We show that the tertiary structure of the SDs is critical for axonal localization of $GABA_{B1a}$. Since the SDs of $GABA_{B1a}$ 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 $GABA_{B1a}$ 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 $GABA_B$ receptors are localized near the active zone (Kulik et al., 2003) it is plausible that $GABA_{B1a}$ 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 $GABA_{B1a}$ partially colocalizes with ER-targeted GFP in the axons, it is possible that some $GABA_{B1a}$ 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 $GABA_{B1a}$ but not $GABA_{B1b}$ to the axonal ER. It is interesting to note that functionally relevant binding sites for the SDs in $GABA_{B1a}$ 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 $GABA_{B1}$ isoforms proposes the existence of dendritic targeting signals in the $GABA_{B1}$ and/or $GABA_{B2}$ 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 $GABA_{B1}$ and $GABA_{B2}$ subunits contain a number of putative dendritic targeting signals. It was recently proposed that $GABA_{B1}$ and $GABA_{B2}$ subunits are transported into dendrites while still residing in the ER and before assembly into heteromeric complexes (Vidal et al., 2007; Ramírez et al., 2009). Consistent with this proposal, we found a colocalization of transfected $GABA_{B1}$ protein with ER-targeted GFP. Possibly, $GABA_{B1}$ and $GABA_{B2}$ 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 $GABA_B$ receptor distribution

Our observation that $GABA_{B1a}$ 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 $GABA_{B1a}$ and $GABA_{B1b}$ subunits are transported into dendrites in somatodendritic post-Golgi carriers. Additionally, some $GABA_{B1a}$ subunits are transported to axons in distinct axonal carriers. Somatodendritic targeting signals, residing within the C-terminal domain of $GABA_{B1}$ and/or $GABA_{B2}$, would sort $GABA_{B1a}$ 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 $GABA_{B1a}$ into axonal transport carriers and explain why much of the $GABA_{B1a}$ 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 $GABA_{B1a}$ and/or $GABA_{B2}$ on the opposite side of the membrane. Our experiments with mGluR1a directly show that luminal SDs can inactivate somatodendritic targeting signals across the membrane, suggesting that they function similarly in the structurally related $GABA_B$ receptors. Of note, conformational changes in the extracellular domain of $GABA_{B1}$ are allosterically coupled to conformational changes in the intracellular domains of $GABA_{B1}$ and $GABA_{B2}$ (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 $GABA_{B1a}$ are a potentially useful experimental tool for delivering transmembrane proteins to axons.

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