The adhesion protein IgSF9b is coupled to neuroligin 2 via S-SCAM to promote inhibitory synapse development

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ynaptic adhesion molecules regulate diverse aspects of synapse formation and maintenance. Many known synaptic adhesion molecules localize at excitatory synapses, whereas relatively little is known about inhibitory synaptic adhesion molecules. Here we report that IgSF9b is a novel, brain-specific, homophilic adhesion molecule that is strongly expressed in GABAergic interneurons. IgSF9b was preferentially localized at inhibitory synapses in cultured rat hippocampal and cortical interneurons and was required for the development of inhibitory synapses onto interneurons. IgSF9b formed a subsynaptic domain distinct from the GABA$_A$ receptor– and gephyrin-containing domain, as indicated by super-resolution imaging. IgSF9b was linked to neuroligin 2, an inhibitory synaptic adhesion molecule coupled to gephyrin, via the multi-PDZ protein S-SCAM. IgSF9b and neuroligin 2 could reciprocally cluster each other. These results suggest a novel mode of inhibitory synaptic organization in which two subsynaptic domains, one containing IgSF9b for synaptic adhesion and the other containing gephyrin and GABA$_A$ receptors for synaptic transmission, are interconnected through S-SCAM and neuroligin 2.

Introduction

Synaptic adhesion molecules regulate synapse formation, maturation, maintenance, and function (Dalva et al., 2007; Biederer and Stagi, 2008; Südhof, 2008; Brose, 2009; Woo et al., 2009; Johnson-Venkatesh and Umemori, 2010; Shen and Scheiffele, 2010; Tallafuss et al., 2010; Williams et al., 2010; Yuzaki, 2010; Siddiqui and Craig, 2011). Many known synaptic adhesion molecules regulate excitatory synapses, whereas relatively little is known about inhibitory synaptic adhesion molecules, which include neuroligin 2 and slitrk3 (Südhof, 2008; Takahashi et al., 2012). Neuroligin 2 selectively localizes to inhibitory synapses and regulates the formation and function of inhibitory synapses (Graf et al., 2004; Varoqueaux et al., 2004; Chih et al., 2005; Levinson et al., 2005; Chubykin et al., 2007). Mouse genetic studies have confirmed that neuroligin 2 regulates GABAergic synapse maturation, inhibitory synaptic transmission, neuronal excitability, retinal signal processing, and anxiety-like behaviors (Varoqueaux et al., 2006; Chubykin et al., 2007; Blundell et al., 2009; Gibson et al., 2009; Hoon et al., 2009; Jedlicka et al., 2011; Poulopoulos et al., 2009).

Abbreviations used in this paper: ANOVA, analysis of variance; DIV, day in vitro; IgSF, immunoglobulin superfamily; mEPSC, miniature excitatory postsynaptic current; mGluR, miniature inhibitory postsynaptic current; PSD, postsynaptic density; S-SCAM, stochastic optical reconstruction microscopy; VGAT, vesicular GABA transporter.

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Neuroligin 2 interacts extracellularly with presynaptic neurexins and triggers inhibitory presynaptic differentiation in contacting axons (Graf et al., 2004; Chih et al., 2005; Levinson et al., 2005). The cytoplasmic region of neuroligin 2 contains two motifs involved in protein–protein interactions. The one located in the middle of the cytoplasmic region directly interacts with gephyrin (Poulopoulos et al., 2009), a major inhibitory postsynaptic scaffold (Fritschy et al., 2008; Fritschy et al., 2012). This interaction is thought to lead to a close apposition of neuroligin 2 with various gephyrin-associated proteins, including GABA$_A$$_1$ receptors and glycine receptors (Fritschy et al., 2008, 2012). Notably, neuroligin 2, but not other neurogins, selectively activates collybistin, a RhoGEF for Cdc42 that binds both gephyrin and the plasma membrane and, thus, leads to the stable tethering of gephyrin to the inhibitory synaptic membrane (Poulopoulos et al., 2009).

The C terminus of neuroligin 2 contains a PDZ-binding motif that interacts with PSD-95 (Irie et al., 1997) and S-SCAM (also known as MAGI-2; Sumita et al., 2007), two postsynaptic scaffolding proteins equipped with multiple domains for protein–protein interactions including PDZ and GK domains (Hirao et al., 1998; Sheng and Kim, 2011). The functional significance of the interaction between neuroligin 2 and PSD-95 remains unclear because these two proteins mainly localize at inhibitory and excitatory synapses, respectively (Sumita et al., 2007; Sheng and Kim, 2011).

S-SCAM, however, distributes to both excitatory and inhibitory synapses, with ~35% of clusters being detected at inhibitory synapses, which is dissimilar to PSD-95 (Sumita et al., 2007). This suggests the possibility that S-SCAM, together with its binding partner neuroligin 2, contributes to the organization of inhibitory synapses. Moreover, highlighting the clinical importance of S-SCAM, chromosomal mutations in the human S-SCAM/MAGI-2 gene have been associated with infantile spasm (also known as West syndrome), an epileptic disorder characterized by seizure, discrete EEG (termed hypersynchrony), and intellectual disability (or mental retardation; Marshall et al., 2008).

Dasm1 (also known as IgSF9; Doudney et al., 2002) is an Ig superfamily (IgSF) adhesion molecule reported to regulate dendritic arborization and excitatory synaptic maturation in hippocampal neurons by knockdown and dominant-negative approaches (Shi et al., 2004a,b), although a more recent study using mice deficient of Dasm1/IgSF9 showed that dendritic arborization in hippocampal neurons from these animals is normal (Mishra et al., 2008). A *Drosophila* homologue of Dasm1 known as Turtle regulates dendritic branching and self-avoidance as well as axonal path finding and self-avoidance (Doudney et al., 2002; Al-Anzi and Wyman, 2009; Ferguson et al., 2009; Long et al., 2009). Dasm1 in mammals has a close relative termed IgSF9b, which shares a similar domain structure with Dasm1; five Ig domains, two fibronectin III (FNIII) domains, one transmembrane domain, and a C-terminal PDZ-binding motif. Importantly, IgSF9b has recently been associated with major depressive disorder (Shyn et al., 2011), but the physiological function of IgSF9b remains unexplored.

In the present study, we found that IgSF9b is selectively expressed in the brain, strongly expressed in GABAergic interneurons, preferentially localized at inhibitory synapses, and required for the development of inhibitory synapses onto interneuronal dendrites. Intriguingly, IgSF9b is localized to a subsynaptic domain distinct from a GABA$_A$$_1$ receptor/gephyrin-containing subsynaptic domain. IgSF9b is linked, via the multi-PDZ protein S-SCAM, to neuroligin 2, which is coupled to gephyrin. These results suggest a novel mode of inhibitory synaptic organization, with detailed molecular mechanisms, where two subsynaptic domains with differential functions, i.e., adhesion and transmission, may act synergistically to develop inhibitory synapses.

**Results**

**IgSF9b is abundantly expressed in GABAergic interneurons**

To understand the function of IgSF9b, a structural homologue of Dasm1 (Figs. 1 A and S1 A), we first generated two different polyclonal antibodies for IgSF9b using two largely independent C-terminal regions of IgSF9b as antigens, where they share minimal amino acid sequence identities with Dasm1/IgSF9 (1593 and 1913; Fig. S1, A and B). These two antibodies revealed essentially identical immunostaining patterns in cultured hippocampal neurons (Fig. S1 C). In addition, a commercially available IgSF9b antibody (HPA010802), raised against another independent region (Fig. S1 B), revealed a similar distribution pattern (Fig. S1 D). The similar distribution patterns of IgSF9b revealed by independent antibodies suggest that the observed IgSF9b signals are authentic.

In cultured hippocampal neurons, IgSF9b signals were detected in punctate clusters in the cell body and dendrite regions of GABAergic interneurons that are positive for the GABA synthetic enzyme glutamic acid decarboxylase GAD67. The majority of GABAergic neurons (~80%) exhibited punctate IgSF9b immunofluorescence ($n = 15$). In contrast, relatively weak signals were detected in some but not all GAD67-negative pyramidal neurons (Fig. 1 B). These results indicate that IgSF9b is more abundant in GABAergic interneurons than in pyramidal neurons.

**IgSF9b is mainly present at inhibitory synapses**

IgSF9b clusters colocalized with the vesicular GABA transporter (VGAT; an inhibitory presynaptic protein) in pyramidal neurons (Fig. 1 C) and in interneurons (Fig. 1 D). IgSF9b also colocalized with gephyrin, an inhibitory postsynaptic protein, and the $\alpha_1$ subunit of GABA$_A$$_1$ receptors in interneurons (Fig. 1 E and F). In contrast, IgSF9b minimally colocalized with the vesicular glutamate transporter vGlut1 and Shank (excitatory pre- and postsynaptic proteins, respectively) in interneurons (Fig. 1 G). In quantitative analysis, ~78% of IgSF9b puncta colocalized with GAD67 ($n = 20$).

In cultured cortical neurons, IgSF9b displayed distribution patterns similar to those observed in hippocampal neurons, being more abundant in GAD65-positive GABAergic interneurons.
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Results. Levels of IgSF9b proteins increased gradually during the first 3 wk of postnatal rat brain development (Fig. 2 D). Similar increases were also observed in dissociated hippocampal neurons (Fig. S2, C–E). IgSF9b was more abundant in synaptic fractions, including crude synaptosomes (P2) and synaptic plasma membranes (LPI), relative to other subcellular fractions. A similar synaptic abundance could also be observed for known inhibitory synaptic proteins including neuroligin 2, gephyrin, and GABA<sub>A</sub> receptor subunits (α1 and γ2; Fig. 2 E).

Within crude synaptosomes, IgSF9b was more abundantly expressed in GAD65-positive interneurons than in GAD65-negative pyramidal neurons, and colocalized with GAD65-positive inhibitory synapses (I and J). IgSF9b colocalizes with gephyrin and GAD65 but not with PSD-95 in dendrites of cortical interneurons. Bars: (B) 20 μm; (C and D, top rows) 20 μm; (C and D, bottom rows) 5 μm; (E–G) 10 μm; (H) 20 μm; (I–L) 5 μm.

Figure 1. IgSF9b is abundant in GABAergic interneurons and mainly present at inhibitory synapses. (A) Domain structure of IgSF9b. SP, signal peptide; Ig, immunoglobulin domain; FNIII, fibronectin type III domain; TM, transmembrane domain; PB, PDZ domain-binding motif. (B) IgSF9b signals are more abundant in interneurons than in pyramidal neurons. Cultured hippocampal neurons (DIV 28, a stage after IgSF9b reaches a plateau of its expression) were labeled by triple immunofluorescence staining for IgSF9b, GAD67, and MAP2. Yellow and red asterisks indicate cell bodies of GABA<sub>A</sub> receptors and GAD67-negative pyramidal neurons, respectively. (C and D) IgSF9b colocalizes with VGAT (an inhibitory presynaptic protein) both in pyramidal neurons (C) and in interneurons (D). The bottom images are enlarged views of the boxed regions. (E–G) IgSF9b colocalizes with gephyrin (E) and the α1 subunit of GABA<sub>A</sub> receptors (F) at VGAT- or GAD65- (an inhibitory presynaptic protein)-positive inhibitory synapses, but not with vGlut1 (an excitatory presynaptic protein)- or Shank (an excitatory postsynaptic protein)-positive excitatory synapses (G) in interneurons. (H–J) In cultured cortical neurons at DIV 28, IgSF9b is more abundantly expressed in GAD65-positive interneurons than in GAD65-negative pyramidal neurons, and colocalizes with GAD65-positive inhibitory synapses (I and J). (K and L) IgSF9b colocalizes with gephyrin and GAD65 but not with PSD-95 in dendrites of cortical interneurons. Bars: (B) 20 μm; (C and D, top rows) 20 μm; (C and D, bottom rows) 5 μm; (E–G) 10 μm; (H) 20 μm; (I–L) 5 μm.

Expression patterns of IgSF9b

We first examined distribution patterns of IgSF9b mRNAs in the rat brain by in situ hybridization. IgSF9b mRNAs were detected in various rat brain regions including cortex, hippocampus, thalamus, olfactory bulb, and cerebellum (Fig. 2 A). In addition to the longest splice variant of IgSF9b, two shorter variants may be expressed based on the database (Fig. S2 A). However, in situ probes directed to the three different mRNA regions revealed similar expression patterns in the mouse brain (Fig. S2 B), which suggests that the two shorter variants, if expressed, are unlikely to be expressed in different cell types.

In Western blot analysis, IgSF9b proteins (~190 kD) were mainly detected in the brain but not in other tissues (Fig. 2 B). IgSF9b proteins were detected in brain regions including cortex, hippocampus, cerebellum, and striatum, as revealed by immunoblot analysis (Fig. 2 C), similar to the in situ hybridization results. Levels of IgSF9b proteins increased gradually during the first 3 wk of postnatal rat brain development (Fig. 2 D). Similar increases were also observed in dissociated hippocampal neurons (Fig. S2, C–E). IgSF9b was more abundant in synaptic fractions, including crude synaptosomes (P2) and synaptic plasma membranes (LPI), relative to other subcellular fractions. A similar synaptic abundance could also be observed for known inhibitory synaptic proteins including neuroligin 2, gephyrin, and GABA<sub>A</sub> receptor subunits (α1 and γ2; Fig. 2 E).

Within crude synaptosomes, IgSF9b was highly enriched in postsynaptic density (PSD) fractions, being detected in PSD III, the most detergent-resistant PSD fraction (Fig. 2 F). This extent of PSD enrichment is comparable to that of PSD-95, an excitatory postsynaptic scaffold protein tightly associated with the PSD. Notably, the γ2 subunit of GABA<sub>A</sub> receptors was also highly detected in PSD III, whereas neuroligin 2 and the α1 subunit of GABA<sub>A</sub> receptors were enriched up to PSD II. These results suggest that IgSF9b, together with the γ2 subunit of GABA<sub>A</sub> receptors, is tightly associated with inhibitory synapses.

Because IgSF9b is a novel membrane protein, we examined glycosylation patterns of IgSF9b. Neuraminidase significantly reduced the size of IgSF9b, whereas O-glycosidase combined with neuraminidase did not further reduce the size of IgSF9b (Fig. 2 G), indicating that IgSF9b is mainly modified by sialylation, but not by O-glycosylation. PNGase F slightly reduced the size of IgSF9b, which indicates that IgSF9b is moderately N-glycosylated.
IgSF9b mediates homophilic adhesion

IgSF9b mediates homophilic adhesion domains (Ig and FNIII) in the extracellular region, and turtle, a Drosophila homologue of IgSF9b and Dasm1/IgSF9, mediates homophilic adhesion (Ferguson et al., 2009). To test whether IgSF9b mediates homophilic adhesion, we incubated HEK293T cells expressing IgSF9b with recombinant, soluble IgSF9b protein (the ectodomain of IgSF9b fused to human Fc; IgSF9b-Ecto-Fc). IgSF9b-Ecto-Fc bound to IgSF9b on HEK293T cells but not to other adhesion molecules, including Dasm1/IgSF9 (Fig. 3 A), which suggests that IgSF9b mediates homophilic adhesion.

In addition, IgSF9b expressed in HEK293T cells accumulated at sites of cell–cell contacts (Fig. 3 B). Moreover, IgSF9b on HEK293T cells induced accumulation of IgSF9b on contacting neurites of cocultured neurons, whereas control HEK293T cells expressing EGFP alone did not (Fig. 3 C). Similar results were obtained in GABAergic interneurons and pyramidal neurons (Fig. S3, A and B). These results suggest that IgSF9b mediates homophilic cell adhesion.

IgSF9b is not sufficient to induce synapse formation

Given that IgSF9b is a homophilic adhesion molecule enriched at inhibitory synapses, IgSF9b might function as a synaptic organizer that induces protein clustering in contacting axons and dendrites. In the coculture (or mixed culture) assay, an assay commonly used to test the capabilities of adhesion molecules to trigger synapse formation (Scheiffele et al., 2000; Biederer and Scheiffele, 2007), IgSF9b-expressing HEK293T cells did not induce clustering of the presynaptic protein synapsin I, VGAT, or vGlut1 in contacting axons of cocultured hippocampal neurons (Fig. 3, D and E; and Fig. S3, C–F). Similarly, IgSF9b did not induce clustering of postsynaptic proteins (gephyrin and PSD-95) in contacting dendrites of GABAergic interneurons and pyramidal neurons (Fig. 3, F–I; and Fig. S3, G and H). These results suggest that IgSF9b lacks the activity to induce synaptic protein clustering in contacting axons and dendrites.

Postsynaptic IgSF9b knockdown reduces the number of VGAT-positive gephyrin clusters in interneurons

Although IgSF9b alone is not sufficient to trigger synapse formation, IgSF9b may still regulate inhibitory synaptic functions. To this end, we generated an IgSF9b shRNA knockdown construct (sh-IgSF9b) coexpressing EGFP, along with a mutant sh-IgSF9b construct that lacks knockdown activity (sh-IgSF9b*) as a control. When expressed in HEK293T cells and cultured hippocampal neurons (day in vitro [DIV] 15–20), sh-IgSF9b substantially reduced IgSF9b expression by ~70% in cultured neurons (Fig. S4, A–D). We expressed sh-IgSF9b or control
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by VGAT-positive gephyrin clusters, compared with empty shRNA vector (sh-vec; negative control; Fig. 4, A and B). In contrast, sh-IgSF9b* had no effect on inhibitory synapse number in interneurons. IgSF9b knockdown had no effect on excitatory synapse number (vGlut1-positive PSD-95 clusters) in interneurons (Fig. 4, C and D). In pyramidal neurons, where weaker IgSF9b expression is observed, sh-IgSF9b caused a tendency constructs in a small fraction of neurons in culture and assessed synapses made onto the somatodendritic domain of these neurons. In this system, the presynaptic inputs arise mainly from surrounding untransfected axons, thus knockdown will reveal roles of postsynaptic IgSF9b.

sh-IgSF9b expressed in interneurons caused a small but significant decrease in the number of inhibitory synapses, defined by VGAT-positive gephyrin clusters, compared with empty shRNA vector (sh-vec; negative control; Fig. 4, A and B). In contrast, sh-IgSF9b* had no effect on inhibitory synapse number in interneurons. IgSF9b knockdown had no effect on excitatory synapse number (vGlut1-positive PSD-95 clusters) in interneurons (Fig. 4, C and D). In pyramidal neurons, where weaker IgSF9b expression is observed, sh-IgSF9b caused a tendency

Figure 3. IgSF9b mediates homophilic adhesion but is not sufficient to induce synapse formation. (A) Homophilic adhesion by IgSF9b. HEK293T expressing IgSF9b-pDis (ectodomain of IgSF9b), or other synaptic adhesion molecules, were incubated with IgSF9b-Ecto-Fc, followed by staining for Fc and the expressed adhesion molecules. (B) IgSF9b accumulates at sites of cell–cell contacts. HEK293T cells were transfected with C-terminally Flag-tagged IgSF9b that lacks the cytosolic region (IgSF9b-Flag), and stained for Flag. (C) IgSF9b-expressing HEK293T cells induce clustering of IgSF9b in contacting neurites of cocultured neurons. HEK293T cells expressing IgSF9b-Flag were cocultured with hippocampal neurons transfected with IgSF9b-pDis (detected with anti-HA). (D–I) IgSF9b-expressing HEK293T cells do not induce clustering of synapsin I (D). gephyrin (F), or PSD-95 (H) in contacting axons or dendrites. HEK293T cells transfected with IgSF9b-pDis, or EGFP alone (negative control), were cocultured with hippocampal neurons (DIV 9–12) and stained for the indicated synaptic proteins (E, G, and I). Quantification of integrated intensities of the synaptic proteins on HEK293T cells normalized to the cell area. au, arbitrary units. Error bars indicate mean ± SEM. n = 15 (E, F, G, and I).

Figure 4. IgSF9b knockdown reduces the number of VGAT-positive gephyrin clusters and the frequency of inhibitory synaptic transmission in interneurons. (A) IgSF9b knockdown reduces the number of inhibitory synapses (VGAT-positive gephyrin) in interneurons. Cultured hippocampal neurons expressing sh-vec, sh-IgSF9b, or sh-IgSF9b* (DIV 15–20) were triply stained for EGFP, gephyrin, and VGAT. Bar, 10 µm. (B) Quantification of the results from A. n = 22; *, P < 0.05, ANOVA and Tukey’s multiple comparison test. (C) IgSF9b knockdown has no effect on the number of excitatory synapses (vGlut1-positive PSD-95 clusters) in interneurons. Bar, 10 µm. (D) Quantification of the results from C. n = 19, ANOVA and Tukey’s multiple comparison test. (E–G) IgSF9b knockdown reduces the frequency (F) but not amplitude (G) of mIPSCs in interneurons. Sample traces of mIPSCs are shown in E. n = 22 for sh-vec and 20 for sh-IgSF9b; *, P < 0.05; Student’s t test. (H–J) IgSF9b knockdown has no effect on the frequency (I) or amplitude (J) of mEPSCs in interneurons. n = 22 for sh-vec and 20 for sh-IgSF9b; Student’s t test. Error bars indicate mean ± SEM.
Contrary to mIPSCs, sh-IgSF9b had no effect on miniature excitatory postsynaptic currents (mEPSCs) in both frequency and amplitude (Fig. 4, H–J). Therefore, postsynaptic IgSF9b is required for normal frequency of inhibitory transmission in interneurons, which is consistent with its role in controlling inhibitory synapse number. 

IgSF9b and gephyrin/GABA 
A receptors display distinct subsynaptic distributions

While searching for mechanisms underlying IgSF9b-dependent inhibitory synapse development, we turned to the observation that IgSF9b and gephyrin often display close but partially non-overlapping localization at GAD65-positive inhibitory synapses (Fig. 5 A). Contrary to mIPSCs, sh-IgSF9b had no effect on miniature excitatory postsynaptic currents (mEPSCs) in both frequency and amplitude (Fig. 4, H–J). Therefore, postsynaptic IgSF9b is required for normal frequency of inhibitory transmission in interneurons, which is consistent with its role in controlling inhibitory synapse number.

Figure 5. Gephyrin colocalizes with neuroligin 2 and the γ2 subunit of GABA 
A receptors, whereas IgSF9b displays close but minimally overlapping localization with gephyrin or the γ2 subunit of GABA 
A receptors. [A] In normal-resolution immunofluorescence imaging, IgSF9b and gephyrin show closely associated but distinct distribution patterns at GAD65-positive inhibitory synapses. Cultured hippocampal neurons (DIV 21) were triply stained for IgSF9b, gephyrin, and GAD65. [B and C] Cultured hippocampal neurons (DIV 21) were visualized by the combinations of double staining indicated and normal (non-STORM) light microscopy. Bars: [A, left] 20 µm; [A, right] 2 µm; [B and C] 1 µm.

to reduce inhibitory synapse number, although it did not reach statistical significance (Fig. S4, E–H). These results suggest that postsynaptic IgSF9b is important for the development of inhibitory synapses in interneurons, which is consistent with the finding that IgSF9b is abundantly expressed in interneurons and is mainly present at inhibitory synapses.

**Postsynaptic IgSF9b knockdown reduces inhibitory synaptic transmission in interneurons**

We next evaluated whether IgSF9b knockdown affects inhibitory synaptic transmission. IgSF9b knockdown reduced the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in interneurons, whereas sh-vec had no effect (Fig. 4, E and F). The amplitude of mIPSCs, however, was unaffected by sh-IgSF9b (Fig. 4, E and G). 

IgSF9b and gephyrin/GABA 
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While searching for mechanisms underlying IgSF9b-dependent inhibitory synapse development, we turned to the observation that IgSF9b and gephyrin often display close but partially non-overlapping localization at GAD65-positive inhibitory synapses in cultured hippocampal neurons (Fig. 5 A). We thus tested more systematically various combinations of double staining...
Because gephyrin and the γ2 subunit of GABA_A receptors are considered to be core components of the inhibitory synapse (Fritschy et al., 2008; Luscher et al., 2011; Fritschy et al., 2012), these results suggest that IgSF9b forms an inhibitory subsynaptic domain that is intimately associated with, but distinct from, the core complex containing GABA_A receptors and gephyrin.

**IgSF9b interacts with S-SCAM at inhibitory synapses**

The close apposition between IgSF9b and GABA_A receptor/gephyrin-containing inhibitory subsynaptic domains suggests that there might be a molecular mechanism linking the two domains. To this end, we searched for proteins that interact with IgSF9b.

In a yeast two-hybrid screen with the IgSF9b C terminus (last 75 aa residues), which contains a PDZ domain-binding motif, we identified MAGI-1 (human), a member of the S-SCAM/MAGI family (Hirao et al., 1998), as a novel binding partner of IgSF9b. Because MAGI-1 has been reported to be absent from inhibitory synapses (Sumita et al., 2007), we focused on S-SCAM...
with its inhibitory synaptic localization. In addition, MAGI proteins formed a complex with neuroligin 2 (Fig. 7 B), which is consistent with the previously reported interaction between S-SCAM and neuroligin 2 (Sumita et al., 2007).

In cultured hippocampal neurons, IgSF9b colocalized with MAGI proteins on dendrites (Fig. 7 C). Furthermore, specifically in GABAergic interneurons, we found that MAGI proteins clustered at GAD67-positive inhibitory synapses, using both an antibody generated in this study (2015; Fig. S5, A and B), and an independent antibody (42574; Fig. S5, C and D). In addition, some MAGI proteins colocalized with vGlut1 clusters in interneurons (Fig. S5 E). To specifically test the localization of MAGI-2, which is highly similar to MAGI-1 and known to be present at inhibitory synapses (Sumita et al., 2007). In an in vitro binding assay, IgSF9b formed a complex with S-SCAM but not with PSD-95 or gephyrin, in heterologous cells. HEK293T cells transfected with IgSF9b (untagged) + tagged S-SCAM, PSD-95, or gephyrin were immunoprecipitated and immunoblotted with the indicated antibodies. The bottom panels indicate immunoprecipitation experiments performed in a reverse orientation. IP, immunoprecipitation; IB, immunoblot.

(A) IgSF9b forms a complex with S-SCAM, but not with PSD-95 or gephyrin, in heterologous cells. HEK293T cells transfected with IgSF9b (untagged) + tagged S-SCAM, PSD-95, or gephyrin were immunoprecipitated and immunoblotted with the indicated antibodies. The bottom panels indicate immunoprecipitation experiments performed in a reverse orientation. IP, immunoprecipitation; IB, immunoblot.

(B) IgSF9b forms a complex with S-SCAM and neuroligin 2, but not with PSD-95, Shank, or neuroligin 1, in the brain. In addition, MAGI proteins form a complex with neuroligin 2. Lysates of the crude synaptosomal fraction from adult rat brains (6 wk) were immunoprecipitated with IgSF9b (left) or pan-MAGI (right) antibodies and immunoblotted. MAGI proteins form a complex with neuroligin 2. (C) IgSF9b colocalizes with MAGI proteins in cultured neurons. Cultured hippocampal neurons at DIV 16 were stained for endogenous IgSF9b, S-SCAM, and MAP2. Arrowheads indicate sites of colocalization. Bar, 10 µm.

(D) Domain structure of S-SCAM. Neuroligin 2– or IgSF9b-binding regions are indicated. PDZ, PSD-95/Dlg/ZO-1 domain; GK, guanylate kinase-like domain.

(E) IgSF9b interacts with S-SCAM at inhibitory synapses.

In an in vitro binding assay, IgSF9b formed a complex with S-SCAM but not with PSD-95 or gephyrin in HEK293T cells (Fig. 7 A). To assess endogenous association of IgSF9b with S-SCAM, we were limited by cross-reactivity of our anti–S-SCAM antibodies with MAGI-1 (Fig. S5, A and B), and thus assessed association of IgSF9b with S-SCAM using these pan-MAGI antibodies. In the brain, IgSF9b coprecipitated with MAGI proteins but not with other postsynaptic scaffolds including gephyrin, PSD-95, and Shank (Fig. 7 B). Notably, IgSF9b formed a weak complex with neuroligin 2 but not with neuroligin 1, which is consistent with its inhibitory synaptic localization. In addition, MAGI proteins formed a complex with neuroligin 2 (Fig. 7 B), which is consistent with the previously reported interaction between S-SCAM and neuroligin 2 (Sumita et al., 2007).

In cultured hippocampal neurons, IgSF9b colocalized with MAGI proteins on dendrites (Fig. 7 C). Furthermore, specifically in GABAAergic interneurons, we found that MAGI proteins clustered at GAD67-positive inhibitory synapses, using both an antibody generated in this study (2015; Fig. S5, A and B), and an independent antibody (42574; Fig. S5, C and D). In addition, some MAGI proteins colocalized with vGlut1 clusters in interneurons (Fig. S5 E). To specifically test the localization
of S-SCAM independent of MAGI-1, we expressed tagged S-SCAM at a low level in cultured neurons. Exogenously expressed EGFP-tagged S-SCAM partially localized at VGAT-positive inhibitory synapses in both interneurons and pyramidal neurons (Fig. S5, F and G). This colocalization is in line with the previous finding that ~35% of S-SCAM clusters are detected at inhibitory synapses in cultured hippocampal neurons (Sumita et al., 2007). Collectively, these data suggest that S-SCAM is present at both excitatory and inhibitory synapses in interneurons, which is consistent with the molecular and biochemical associations.

We next mapped minimal IgSF9b-binding domains in S-SCAM. Among the three deletion variants of S-SCAM tested (PDZ0–GK, WW–PDZ1, and PDZ2–5), PDZ2–5 was sufficient for IgSF9b binding (Fig. 7, D–F). WW–PDZ1 interacted with neuroligin 2, as previously reported (Sumita et al., 2007). Experiments with additional deletion variants of S-SCAM revealed that PDZ4–5 of S-SCAM is sufficient for IgSF9b binding (Fig. 7 G).

**S-SCAM bridges IgSF9b and neuroligin 2**

Because two distinct regions of S-SCAM bind IgSF9b and neuroligin 2 (Figs. 7 D and 8 A), we tested whether the three proteins can form a ternary complex. In heterologous cells, IgSF9b coprecipitated with neuroligin 2 only in the presence of S-SCAM (Fig. 8 B), which is consistent with the coprecipitation of IgSF9b with neuroligin 2 and MAGI proteins in the brain (Fig. 7 B). In addition, IgSF9b colocalized with both MAGI proteins and neuroligin 2, but not with PSD-95, in cultured interneurons (Fig. 8 C). These results suggest that IgSF9b forms a ternary complex with S-SCAM and neuroligin 2 at inhibitory synapses.

To see if S-SCAM is critically involved in linking IgSF9b and neuroligin 2 clusters in neurons, we tested whether S-SCAM knockdown decreases the colocalization between IgSF9b and neuroligin 2. We found that S-SCAM knockdown (sh-S-SCAM, DIV 15–20) in cultured interneurons suppressed the colocalization between IgSF9b and neuroligin 2, whereas it had no effect on the number of single IgSF9b or neuroligin 2 clusters (Fig. 8, D–G). A mutant knockdown construct that lacks the knockdown activity (sh-S-SCAM*) had no effect on the colocalization between IgSF9b and neuroligin 2. In addition, S-SCAM knockdown decreased the number of VGAT-positive gephyrin clusters (Fig. 8, H and I). These results suggest that S-SCAM is required for the close association between IgSF9b- and neuroligin 2-containing subsynaptic domains in interneurons, and that S-SCAM is required for the development or maintenance of inhibitory synaptic structures containing gephyrin and VGAT.

**IgSF9b and neuroligin 2 bidirectionally drive protein clustering**

The formation of the ternary complex between IgSF9b, S-SCAM, and neuroligin 2 may be driven unidirectionally by IgSF9b or neuroligin 2, or bidirectionally. To test this idea, HEK293T cells triply expressing IgSF9b, S-SCAM, and neuroligin 2 were incubated with preclustered neurexin β1 ectodomain to induce neuroligin 2 aggregation. This aggregation induced colocalization of IgSF9b only in the presence of S-SCAM in heterologous cells (Fig. 9 A). In cultured hippocampal neurons treated with preclustered neurexin β1, primary clustering of endogenous neuroligin 2 induced coclustering of MAGI proteins and IgSF9b at subcellular sites lacking VGAT (Fig. 9, B and C).

Conversely, in cultured neurons expressing N-terminally EGFP-tagged IgSF9b, direct aggregation of IgSF9b by GFP antibody–coated beads induced coclustering of endogenous neuroligin 2 at sites that lack synapsin I (Fig. 9, D and E). These results suggest that the formation of the ternary complex containing IgSF9b, S-SCAM, and neuroligin 2 can be driven bidirectionally, initiating from neuroligin 2 or IgSF9b.

**Neuroligin 2 does not affect the general stability or synaptic localization of IgSF9b**

IgSF9b and neuroligin 2, which can bidirectionally drive protein clustering, may also regulate the synaptic localization or general stability of each other. However, neuroligin 2 deletion in mice, which has been reported previously (Varoqueaux et al., 2006), had no effect on overall levels of IgSF9b, as determined by immunoblot analysis of whole-brain lysates (Fig. 9, F and G). In addition, neuroligin 2 deficiency had no effect on synaptic levels of IgSF9b, as determined by immunoblot analysis of synaptic brain fractions including synaptosomal membranes and PSD fractions where IgSF9b is highly enriched (Fig. 9, F and G). These results suggest that neuroligin 2 has minimal influences on overall stability or synaptic localization of IgSF9b.

**Gephyrin can be clustered by neuroligin 2 but not by IgSF9b**

Because IgSF9b can drive coclustering of neuroligin 2 (Fig. 9, D and E), we tested whether IgSF9b can also drive coclustering of gephyrin, an inhibitory postsynaptic scaffold that directly interacts with neuroligin 2 (Pouloupolous et al., 2009) and diverse inhibitory postsynaptic proteins including GABA_A and glycine receptors and signaling, cytoskeletal, and trafficking proteins (Fritschy et al., 2008). However, bead aggregation of IgSF9b in cultured neurons did not induce coclustering of gephyrin (Fig. 10, A and B), despite the fact that it could cocluster neuroligin 2 (Fig. 10 C). In contrast, bead aggregation of neuroligin 2 could induce coclustering of gephyrin at VGAT-negative sites, whereas CD4 (a negative control) could not (Fig. 10, A and B). Similarly, neuroligin 2 aggregation by preclustered neurexin β1 induced gephyrin coclustering (Fig. 9 B). These results suggest that gephyrin can be clustered by neuroligin 2 but not by IgSF9b.

**Discussion**

**IgSF9b, a novel inhibitory synaptic adhesion molecule**

In the present study, we have identified IgSF9b as a novel inhibitory synaptic adhesion molecule.

IgSF9b differs from Dasm1/IgSF9 in several aspects. Dasm1 is mainly localized at and regulates excitatory synapses (Shi et al., 2004a,b), whereas IgSF9b mainly regulates inhibitory synapses. In addition, Dasm1 does not associate with IgSF9b (Fig. 3 A), despite the fact that they show substantial amino acid identity in
Their extracellular domains (Fig. S1A). Given that their cytoplasmic domains are quite dissimilar in amino acid sequences, we predict that IgSF9b and Dasm1 have distinct intracellular partners mediating different intracellular signaling pathways.

IgSF9b, to the best of our knowledge, represents the fourth adhesion molecule that is selectively present at inhibitory synapses, next to neuroligin 2, dystroglycan, and slitrk3 (Lévi et al., 2002; Varoqueaux et al., 2004; Fritschy et al., 2012). In culture, IgSF9b contributes mainly to inhibitory synapses onto GABAergic interneurons, onto the majority if not all GABAergic neurons (Fig. 1), and is thus not selective for interneuron type. Expression patterns in vivo also suggest selective function of IgSF9b in interneurons, although some levels of expression are also observed in cortical and hippocampal CA1 pyramidal cells and cerebellar granule cells. Our study did not explore how IgSF9b contributes to interneuronal circuitry in the brain. However, in dissociated hippocampal cultures, knockdown of postsynaptic IgSF9b in GABAergic interneurons suppresses inhibitory synapse number and synaptic transmission onto these neurons, whereas the same...
knockdown in pyramidal neurons has no effect (Fig. 4 and Fig. S4). If IgSF9b has a similar function in vivo, it could be an interesting general regulator of inhibitory transmission selectively among GABAergic neurons. Although dystroglycan also localizes to a subset of GABAergic synapses (Lévi et al., 2002) and neuroligin 2 may contribute functionally to specific subsets of GABAergic synapses (Gibson et al., 2009; Poulopoulos et al., 2009), this selectivity of IgSF9b for GABAergic synapses onto GABAergic neurons appears unique.

Second, IgSF9b is distinct in that it is a homophilic cell adhesion molecule (Fig. 3), whereas neuroligin 2, dystroglycan, and slitrk3 are not. Based on this feature, it is likely that the synaptic IgSF9b observed here represents both presynaptic and postsynaptic pools. Given that postsynaptic knockdown of IgSF9b reduces inhibitory synapse number and function in GABAergic interneurons, postsynaptic IgSF9b appears to be important. We suspect that IgSF9b may act from the presynaptic side as well, through trans-synaptic homophilic adhesion, although additional experiments will be required to explore this possibility.

Third, IgSF9b is not sufficient to trigger synapse formation (Fig. 3), whereas neuroligin 2 and slitrk3 have such activities. The developmental expression of IgSF9b also lags behind that of neuroligin 2 (Fig. 2), and IgSF9b appears at inhibitory synapses in hippocampal cultures at a late stage of their development (Fig. S2). Therefore, IgSF9b may stabilize the inhibitory synapses initially organized by other adhesion molecules. Lastly, IgSF9b can be cosegregated with postsynaptic neurexin via neuroligin 2, whereas dystroglycan is not (Graf et al., 2004), which suggests that IgSF9b is an inhibitory adhesion molecule closely associated with the neuroligin–neurexin system.

Subsynaptic domains at inhibitory synapses
Our super-resolution data indicate that IgSF9b and gephyrin/GABA<sub>A</sub> receptors (γ2) distribute to two distinct inhibitory
as previously suggested for predetermined postsynaptic hot-spots for development of functional excitatory synapses (Gerrow et al., 2006) and for rapid and efficient assembly of presynaptic active zones (Zhai et al., 2001; Shapira et al., 2003).

S-SCAM as an adaptor bridging IgSF9b and neuroligin 2

Our data suggest that S-SCAM is important for physical coupling of the two inhibitory subsynaptic domains. In support of this notion, S-SCAM forms a ternary complex with IgSF9b and neuroligin 2 (Fig. 8). Moreover, S-SCAM knockdown enhances mislocalization of IgSF9b and neuroligin 2 (Fig. 8). What would be the advantages of S-SCAM as a bridging molecule?

S-SCAM is a protein with multiple domains for protein–protein interactions (i.e., six PDZ domains) and many known binding partners. Therefore, S-SCAM may be able to function as a focal site of multiprotein interaction and assembly that would support a dynamic and regulated association between the two subsynaptic domains. Known S-SCAM–binding proteins thus far include neuroligin 1, neuroligin 2, NMDA receptors, GKAP/SAPAP, and SynArfGEF (Hirao et al., 1998; Kim and Sheng, 2004; Fukaya et al., 2011), where only neuroligin 2 and SynArfGEF are inhibitory synaptic proteins. SynArfGEF, a guanine nucleotide exchange factor for the Arf6 small GTPases, is preferentially localized at inhibitory synapses, and directly interacts with dystrophin/utrophin (Fukaya et al., 2011), which, together with dystroglycan, are components of the dystrophin-associated glycoprotein complex. Therefore, S-SCAM has the potential to regulate Arf-dependent synaptic membrane/protein trafficking at the junction between the two subsynaptic domains, and to position the dystrophin-associated glycoprotein complex close to this junction.
This concept of a multidomain connector for neighboring macromolecular structures is reminiscent of the finding that dynamin-3, a GTPase with multiple domains for protein–protein interactions, positions the endocytic zone, an excitatory postsynaptic hotspot for local AMPA receptor endocytosis (Blanpied et al., 2002), close to the PSD via its direct interaction with Homer, a PDZ and EVH1 domain–containing adaptor protein in the PSD (Lu et al., 2007).

Bidirectional protein clustering by IgSF9b and neuroligin 2

As in excitatory synapses, molecular organization of inhibitory postsynaptic protein complexes involves key synaptic scaffolding proteins such as gephyrin (Fritschi et al., 2008, 2012; Tyagarajan and Fritschy, 2010; Sheng and Kim, 2011). Gephyrin forms homomultimers and interacts with diverse postsynaptic membrane, signaling, and cytoskeletal proteins. This gephyrin-based postsynaptic protein assembly is thought to be initiated by the clustering of neurolin 2 driven by presynaptic neu-rexins (Craig and Kang, 2007; Südhof, 2008; Shen and Scheiffele, 2010; Krueger et al., 2012). Our results extend this view of inhibitory postsynaptic assembly. Specifically, initial clustering of neurolin 2 appears to induce coclustering of S-SCAM and IgSF9b, in addition to gephyrin, in order to strengthen synaptic adhesion.

Therefore, functions of neurolin 2 as a postsynaptic organizer at inhibitory synapses appear to be multifold. They are to induce clustering of (1) gephyrin and associated proteins for postsynaptic differentiation, (2) S-SCAM and IgSF9b for lateral association with a subsynaptic adhesive domain, and (3) presynaptic neu-rexins for presynaptic differentiation.

Our data suggest that IgSF9b and neurolin 2 can reciprocally cluster each other (Fig. 9). Possible functions of neurolin 2 clustering by IgSF9b are unclear. Postsynaptic assembly in this direction may have a limited impact given the findings from the artificial aggregation assays that IgSF9b drives protein clustering up to neurolin 2 but not beyond to gephyrin, whereas neurolin 2 can cluster gephyrin in addition to IgSF9b. However, the bidirectional clustering between IgSF9b and neurolin 2 may enhance the chance of rate or force of forming the connection between the two subsynaptic domains. Altogether, the reciprocal clustering of IgSF9b and neurolin 2 is likely to enhance the development and stabilization of inhibitory synapses, particularly in interneurons where IgSF9b is abundant.

In conclusion, our data identifies a novel inhibitory synaptic adhesion molecule IgSF9b that localizes to a distinct subsynaptic domain and is coupled to neurolin 2 via S-SCAM to promote inhibitory synaptic development. A direction for future studies would be to explore the functional significance of the two subsynaptic domains, and related regulatory mechanisms. The selective localization and function of IgSF9b at inhibitory synapses onto GABAergic interneurons, at least in culture, places IgSF9b in a unique position among inhibitory synaptic adhesion proteins and identify it as a potential drug target. Paradoxically, we predict that down-regulation of IgSF9b level or function, through selectively reducing inhibition onto GABAergic neurons, may increase GABAergic transmission to pyramidal cells and reduce overall circuit excitation.

Materials and methods

DNA constructs

Full-length mouse IgSF9b (accession no. NM_001129787, original GenBank version, aa 1–1,441) was amplified by PCR from a mouse brain cDNA library (BD) and subcloned into pEGFP-N1 (Takara Bio Inc.) with an intact stop codon at the 3′ end. Untagged IgSF9b was generated by subcloning of full-length IgSF9b (aa 1–1,441) into pCwW1 (British Biotech). Mcx epitope (EQUKLISEEDL) and GFP was inserted between residues 28 and 29 of IgSF9b in pCwW1 and pEGFP-N1, respectively. Epitope-domains of IgSF9b (aa 1–716) were subcloned into pEGFP-N1 in which GFP was replaced with human Fc and Display (Intragen). The C terminally Flag-tagged IgSF9b that lacks a cytosolic region (aa 1–756) was subcloned into pEGFP-N1, which has triple Flag motifs with a stop codon. For the yeast two-hybrid assay, the last 75 aa of IgSF9b were subcloned into pBHA (vector bait). For siRNA knockdown, nucleotides 216–234 (5′-CATCAAGTTTGGCCTATACT-3′) of rat IgSF9b, its point mutant (5′-CATAAAGTCGCCCTACTAT-3′), nucleotides 1,328–1,346 (5′-GAGATGAGCGGCGACAGTGT-3′) of rat S-SCAM, and its point mutant (5′-GAGACGACCTGAGTAGGGT-3′) were selected and subcloned into pSuper.gfp/neol [OligoEngine]. For N-terminally EGFP tagged S-SCAM, full-length rat S-SCAM (GenBank accession no. NM_033621, aa 1–1,277) was amplified from untagged S-SCAM and subcloned into pEGFP-C1 (Takara Bio Inc.). The following deletion variants of S-SCAM were also subcloned into pEGFP-C1: PDZS0-GK (aa 1–301), WW-PDZ1 (aa 296–578), PDZ2S (aa 573–1,277), PDZ1-2 (aa 417–710), PDZ2-3 (aa 573–892), PDZ3-4 (aa 747–1,028), and PDZ4-5 (aa 867–1,277). For N-terminally EGFP tagged MAG1-1, full-length rat MAG1-1 (NM_01300045, aa 1–1,255) was amplified from a rat brain cDNA library (BD) and subcloned into pEGFP-C1. The following expression constructs have been described previously: YFP-gephyrin (rat gephyrin isoform 2.6 was subcloned into modified p lentinLox.P7; Dobie and Craig, 2011), HA-NL2G2 (a gift from P. Scheiffele, Biozentrum of the University of Basel, Basel, Switzerland; mouse neur-ulin-2 was subcloned into pNICE; Scheiffele et al., 2000), YFP-Cd4 (to mature form of Cd4 was subcloned into spYFP-C1; Takahashi et al., 2011), YFP-NL2G2 (modified from HA-NL2G; Graf et al., 2004), untagged S-SCAM (from Y. Hata, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan; full-length rat S-SCAM was subcloned into pcMV5; Hiroa et al., 1998), and NRx1-1-Fc (pCMVBgN1B1–3 from T. Sudhof, Stanford University Medical School, Stanford, CA; aa 1–300 of rat neurini-1β lacking an insert in splice site #4 was subcloned into pCMV-G, Ko et al., 2009).

Antibodies

Guinea pig (1593) and rabbit (1913) polyclonal IgSF9b antibodies were raised against GST-IgSF9b aa 1,126–1,351 (226 aa) and aa 1,293–1,441 (149 aa), respectively. GST–S-SCAM aa 303–405 (103 aa, WW domains) was used as an immunogen to generate guinea pig (2012) and rabbit (2013) polyclonal S-SCAM antibodies. The following antibodies have been described previously: EGFP (1432, guinea pig, H6-EGFP aa 1–240; Choi et al., 2006), PSD-95 (SM55, rabbit, H6-PSD-95 aa 77–299; Choi et al., 2002), neurolin 2 (rabbit, RGGGVGADPAEALRPACP; Takahashi et al., 2011), YFP-NLG2 (modified from HA-NL2G; Graf et al., 2004), untagged S-SCAM (from Y. Hata, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan; full-length rat S-SCAM was subcloned into pcMV5; Hiroa et al., 1998), and NRx1-1-Fc (pCMVBgN1B1–3 from T. Sudhof, Stanford University Medical School, Stanford, CA; aa 1–300 of rat neurin-1β lacking an insert in splice site #4 was subcloned into pCMV-G, Ko et al., 2009).

Deglycosylation assay

Enzymatic deglycosylation was performed with rat P2 (crude synaptic) fractions extracted with 1% deoxycholate (DOC) using N-glycosidase/F (New England Biolabs, Inc.), sialidase/neuraminidase (Roche), and O-glycosidase/endo-α-N-acetylgalactosaminidase (EMD Millipore) according to the manufacturer’s instructions.

In situ hybridization analysis

In situ hybridization was performed as described previously (Kim et al., 2003). In brief, adult rats and mice brains (6 wk) were rapidly extracted.
Brain fractionation and coimmunoprecipitation
Subcellular and PSD fractions of adult rat and mouse brains were prepared as described previously (Carlin et al., 1980; Huttner et al., 1983). In brief, rat brain homogenates (H) were centrifuged at 10,000 g to obtain a crude synaptosome fraction (P2), and the supernatant (S2) was further centrifuged at 160,000 g to obtain light membranes (P3) and cytosol fractions (S3). In parallel, the P2 fraction was subjected to hypotonic lysis and centrifuged at 25,000 g to precipitate synaptosomal membranes (LP1). The supernatant (LS1) was further centrifuged at 160,000 g to obtain a crude synaptic vesicle-enriched fraction (LP2) and soluble fraction (LS2). To obtain PSD fractions, the synaptosomal fraction was extracted with detergents, once with Triton X-100 (PSD I), twice with Triton X-100 (PSD II), and once with Sarkosyl (PSD III). Protein loading was quantified using the Pierce Reversible Protein Stain kit (Thermo Fisher Scientific). For in vivo coimmunoprecipitation, the P2 fraction of adult rat brain was extracted in buffer containing 1% sodium deoxycholate and 50 mM Tris-HCl, pH 9.0, followed by incubation with immunoprecipitation antibodies. For coimmunoprecipitation in heterologous cells, cells were extracted in phosphate buffered saline containing 1% Triton X-100.

Coculture assay
Coculture assays were performed as described previously (Kim et al., 2006; Biederer and Scheiffele, 2007). In brief, primary hippocampal neuron cultures at DIV 10 prepared from embryonic day 18–19 rats were cocultured with HEK293T cells expressing either siGF9b or EGFP. The cocultured cells were immunostained with synapsin1, PSD-95, or gephyrin antibodies.

Neuron culture, transfection, and immunocytochemistry
Primary hippocampal neuronal cultures were prepared from embryonic day 18–19 rat hippocampi as described previously (Goslin and Banker, 1991). Cultures were plated on coverslips coated with poly-lysine and laminin, and grown in neurobasal medium supplemented with B27 (Invitrogen) and 2% fetal bovine serum (Gibco) in a 10% CO2-laminin, and grown in neurobasal medium supplemented with B27 (Invitrogen). For the application of preclustered neurexin1, 0.1% glutaraldehyde, and primary antibodies (2.5 µg/ml anti-gephyrin, 2 µg/ml rabbit anti-lgGF9b, and 2.5 µg/ml guinea pig anti-GABAα-R x2). Secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) were labeled with a mixture of amine-reactive activators and reporters. Alexa Fluor 405 (Invitrogen) and Cy3 (GE Healthcare) were used as the activators, and Alexa Fluor 647 (Invitrogen) was used as the reporter. Concentrations of reactive dyes were controlled such that each antibody had, on average, two activator molecules and 0.6–0.8 reporter molecules.

Imaging was done as described previously (Huang et al., 2008). In brief, STORM images were captured by a super-resolution microscope (N-STORM; Nikon) using a 100x, 1.4 NA objective lens and an EM charge-coupled device camera (Ixon3 897; Andor Technology) at room temperature in a standard imaging buffer that contains 50 mM Tris, pH 7.5, 10 mM NaCl, 0.5 mg/ml glucose oxidase, 40 µg/ml catalase, 10% (wt/vol) glucose, and 0.1 M mercaptoethanol. Two activation lasers and one imaging laser are individually shuttered and illuminate the sample in total internal reflection fluorescence mode. An objective positioner (NANO F-100; Mad City Labs Inc.) was used to stabilize the focusing of the microscope. For two-color imaging, a similar repetitive sequence of activation imaging (1 frame of activation followed by 2 frames of imaging) was used with two activation lasers. 405 nm light from the CUBE laser (Coherent) was used to activate the A405-A647 pair, and S61-nm light from a solid state laser (SAPPHIRE; Coherent) was used to activate the Cy3-A647 pair. A 647-nm laser from the Crystalab (DL647-100) was used for imaging. The localizations were later color-coded based on whether the A647 emission was detected after the 405-nm or 647-nm excitation light. Typical laser powers used for STORM imaging were 60 mW for the imaging laser and ~2 µW–5 µW for each of the activation lasers. A typical STORM image cycle was repeated ~15–20,000 times until eventually fluorescence bleached. We used NIS-Elements software (Nikon) for correction of image drift and cross-talk statistically. For two-color STORM images, each localization was assigned as one point in the STORM image and was false-colored according to the color of the activation laser pulse.

Electrophysiology
Cultured hippocampal neurons were transfected with sh-lgGF9b (Div 15–20), and interneuron- and pyramidal-shaped cells, as defined by morphology (Benson et al., 1994), were whole-cell voltage-clamped at ~60 mV using an Axopatch 200B amplifier (Molecular Devices). To ensure that morphologically identified cells were GABAergic interneurons, we marked cells with bicucullin infusion followed by GAD67 staining, and found that ~93% of the cells were GAD67-positive (n = 15). The extracellular solution contained (in mM): 145 NaCl, 2.5 KCI, 10 Hepes, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 10 glucose, and 0.4 sodium ascorbate. For mIPSC, the intracellular solution contained (in mM): 135 KCl, 10 Hepes, 4 NaCl, 4 Mg-ATP, 0.3 Na-GTP, and 0.5 EGTA. For mEPSC, the intracellular solution contained (in mM): 135 KCl, 10 Hepes, 4 NaCl, 4 Mg-ATP, 0.3 Na-GTP, and 0.5 EGTA. For mEPSC, the intracellular solution contained (in mM): 100 Kglutonate, 20 KCl, 10 Hepes, 8 NaCl, 4 Mg-ATP, 0.3 Na-GTP, and 0.5 EGTA. During measurement, 1 µM TTX, 50 µM A PS, and 10 µM NBQX (all from Tocris Bioscience) for mEPSC or TTX and bicuculline (10 µM, Tocris) for mIPSC were added into the extracellular solution. Synaptic currents were analyzed using a custom-written macro in Igor Pro (WaveMetrics).

Online supplemental material
Fig. S1 shows amino acid sequence alignment of lgGF9b and Dasm1/1-lgGF9f, and generation and characterization of lgGF9b antibodies. Fig. S2 shows distribution of lgGF9b in hippocampal neurons and its independence in situ hybridization probes, and gradually increasing levels of lgGF9b proteins in cultured hippocampal neurons. Fig. S3 shows that lgGF9b expressing HEK293T cells induce clustering of lgGF9b in contacting neurites of co-cultured GAD67-positive interneurons and GAD67-negative pyramidal neurons, but do not induce clustering of VGAT or vGlut1 in contacting axons of excitatory pyramidal neurons. Fig. S4 shows characterization of lgGF9b knockdown constructs, inhibitory interneurons were distinguished from excitatory pyramidal neurons by immunostaining with GABAergic neuronal markers such as GAD67 and/or by morphological aspects such as polygonal shaped cell bodies, less spiny and nontapered dendrites with longer segments between branch points (Benson et al., 1994). To determine the density of synaptic protein clusters, every primary dendritic segment encompassing 50 µm from cell bodies of each neuron was analyzed. For quantification of images from confocal images, objects were thresholded and the integrated intensity of the clusters on transfected HEK293T cells was normalized to the cell area. All values are presented as mean ± SEM and analyzed by Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test.
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