SynCAMs Organize Synapses through Heterophilic Adhesion

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Synapses are asymmetric cell junctions with precisely juxtaposed presynaptic and postsynaptic sides. Transsynaptic adhesion complexes are thought to organize developing synapses. The molecular composition of these complexes, however, remains incompletely understood, precluding us from understanding how adhesion across the synaptic cleft guides synapse development. Here, we define two immunoglobulin superfamily members, SynCAM 1 and 2, that are expressed in neurons in the developing brain and localize to excitatory and inhibitory synapses. They function as cell adhesion molecules and assemble with each other across the synaptic cleft into a specific, transsynaptic SynCAM 1/2 complex. Additionally, SynCAM 1 and 2 promote functional synapses as they increase the number of active presynaptic terminals and enhance excitatory neurotransmission. The interaction of SynCAM 1 and 2 is affected by glycosylation, indicating regulation of this adhesion complex by posttranslational modification. The SynCAM 1/2 complex is representative for the highly defined adhesive patterns of this protein family, the four members of which are expressed in neurons in divergent expression profiles. SynCAMs 1, 2, and 3 each can bind themselves, yet preferentially assemble into specific, heterophilic complexes as shown for the transsynaptic SynCAM 1/2 interaction and a second complex comprising SynCAM 3 and 4. Our results define SynCAM proteins as components of novel heterophilic transsynaptic adhesion complexes that set up asymmetric interactions, with SynCAM proteins contributing to synapse organization and function.

Key words: SynCAM; CADM; synapse; synaptic adhesion; synaptogenesis; synaptic transmission

Introduction

Synapse organization in the CNS requires multiple specification steps to ensure synaptic function. These steps define nascent presynaptic and postsynaptic sites, support the distinct formation of excitatory or inhibitory synapses, and allow proper targeting of neurons. The development of synapses involves transsynaptic interactions of dedicated synaptic adhesion molecules (Scheiffele, 2003; Yamagata et al., 2003; Waites et al., 2005; Akins and Biederer, 2006). These adhesion systems likely comprise the protein complexes spanning the synaptic cleft (Lucic et al., 2005).

Despite the importance of synapse organization for brain development and function, our understanding of synaptic adhesion systems is limited. In both vertebrates and invertebrates, the diverse family of cadherin proteins is perhaps best understood to regulate synapses and neuronal signaling through transsynaptic interactions (Latefi and Colman, 2007; Takeichi, 2007). A different small family of synaptic adhesion molecules was identified in vertebrates with the purification of neurexins (Ushkaryov et al., 1992; Ushkaryov and Südhof, 1993) and their postsynaptic partners, neuroligins (Ichtchenko et al., 1995; Boucard et al., 2005). Neuroligins were shown to interact with neurexins to control the number of functional presynaptic terminals (Scheiffele et al., 2006; Dean et al., 2003; Levinson et al., 2005; Taniguchi et al., 2007). Conversely, neurexins cause postsynaptic protein assembly through binding of neuroligins (Graf et al., 2004; Chih et al., 2005). In vivo, neurexins and neuroligins affect synapse maturation, synaptic transmission, and network function (Missler et al., 2003; Varoqueaux et al., 2006).

In addition, various members of the Ig superfamily have been implicated as synapse organizing molecules. At the Drosophila neuromuscular junction, the adhesion molecule Fasciclin II stabilizes and patterns synapse formation (Davis et al., 1997). In the mollusk Aplysia, aPCAM (Aplysia cell adhesion molecule) contributes to synaptic plasticity (Mayford et al., 1992), and in the nematode Caenorhabditis elegans, the epithelial protein synaptogenesis abnormal-2 (SYG-2) and the neuronal SYG-1 guide synapse formation (Shen and Bargmann, 2003; Shen et al., 2004). In vertebrates, the Ig protein NCAM (neuronal cell adhesion molecule) regulates synapse formation and synaptic plasticity, whereas L1 participates in specifying synaptic sites, and the interactions of Sidekick family members contribute to synapse specification (Yamagata et al., 2003; Gerrow and El-Husseini, 2006).

Recently, the Ig superfamily member SynCAM 1 (synaptic cell...
adhesion molecule 1) was identified as synaptic cell adhesion molecule in vertebrates (Biederer et al., 2002). It is encoded by the gene CADMI (cell adhesion molecule 1) and has also been named TSLC1, Necl-2 (nectin-like molecule 2), and RA175, reflecting its identification in other contexts (Kuramochi et al., 2001; Urase et al., 2001; Shingai et al., 2003). SynCAM 1 contains three extracellular Ig-like domains followed by a single transmembrane region and a short cytosolic sequence that interacts with PDZ [postsynaptic density-95 (PSD-95)/Discs large/ zona occludens-1] domains of synaptic scaffolding molecules (Biederer et al., 2002). SynCAM 1 can engage in a homophilic interaction through its Ig-like domains to mediate cell adhesion (Biederer et al., 2002). In cultured hippocampal neurons, SynCAM 1 overexpression promotes excitatory neurotransmission. Furthermore, presentation of SynCAM 1 from non-neuronal cells to hippocampal neurons drives the neurons to develop fully functional excitatory presynaptic terminals at sites of contact (Biederer et al., 2002; Sara et al., 2005).

To advance our understanding of SynCAM 1 activity at synapses, we defined the transnaptic adhesion complexes in which it participates. Guided by our recent analysis of the four SynCAM family members that share the same domain organization (Biederer, 2006), we considered them as candidate heterophilic binding partners of SynCAM 1. However, expression profiles and interaction patterns of the four SynCAM proteins as well as shared functions in brain have not been described. Specifically, it was not known whether they together confer synaptic adhesion and can contribute to synapse organization and function.

We here identify SynCAMs as a neuronal family of adhesion molecules that prefer heterophilic over homophilic interactions, enabling them to cooperate in synapse organization. SynCAM 1 and 2 assemble into a synaptic adhesion complex, and both proteins affect the organization and function of synapses. SynCAM 3 and 4 constitute the second strong heterophilic pair within the SynCAM family. The adhesion complex of SynCAM 1 and 2 is stable in synaptic membranes in vivo and its components recruit presynaptic proteins and promote excitatory neurotransmission in vitro. Together, our findings identify SynCAM proteins as components of an asymmetric transnaptic adhesion system and introduce a novel molecular mechanism that can contribute to the diversity of transsynaptic interactions and guide synapse organization in the CNS.

**Materials and Methods**

*Antibodies.* Specific antibodies against SynCAM 1 (YUC8) were raised in chicken against the natively N-deglycosylated extracellular sequence of mouse SynCAM 1 expressed as human IgG1 fusion in COS 7 cells. Antibodies against SynCAM 2 (YU524) and SynCAM 3 (YU525) were raised in rabbits against the peptides CKDVKYLKEEDANRKT and CHG-DQTRIQEDPNGKT, respectively, corresponding to amino acid sequences in the second Ig-like domain of the mouse proteins with an N-terminal cysteine for coupling. Antibodies against SynCAM 4 (YU591) were raised in rabbits against the peptide CCGGDKHRKREFFI, corresponding to the C-terminal sequence of the mouse protein with an N-terminal cysteine for coupling. All polyclonal antibodies were affinity purified using standard procedures (Harlow and Lane, 1999). For simultaneous detection of SynCAM 1–3, we used a pleioSynCAM antibody (T2412) raised in rabbits against the SynCAM 1 C-terminal sequence described previously (Biederer et al., 2002) that also recognizes this conserved sequence in SynCAM 2 and 3, but not 4 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). For immunoprecipitation of SynCAM 1 and selected immunoblots as described in the figures, monoclonal antibodies raised in chicken against its extracellular domain were used (clone 3E1; MBL Laboratories, Nagoya, Japan) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Antibodies to synaptotagmin 1 (for immunoblotting, 41.1; for synaptic vesicle uptake, 604.1), synaptophysin (7.2), synapsin (1006002/E028), gephyrin (147011), and GDP dissociation inhibitor (GDI) (81.2) were obtained from Synaptic Systems (Gottingen, Germany); to PSD-95 (MA1–045) from Affinity Bioreagents (Golden, CO); to N-cadherin from BD Biosciences (San Jose, CA); to flag (M2) from Sigma (St. Louis, MO); to and NeuN (neuronal-specific nuclear protein) (MAB377), vGlut1 (AB5905), vGlut2 (AB5907), and GAD65 (AB5082) from Millipore (Billerica, MA). Monoclonal antibodies to actin (JLA20; developed by Jim Jung-Ching Lin, University of Iowa, Iowa City, IA) and SV2 (developed by Kathleen Buckley, Harvard Medical School, Boston, MA) were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Antibodies to valosin-containing protein (VCP) (Sugita and Sudhof, 2000) were a gift from Dr. Thomas Sudhof (University of Texas Southwestern Medical Center, Dallas, TX); antibodies to Nefrin-G ligand (NI) (Kim et al., 2006) were a gift from Dr. Eunjoon Kim (KAIST, Republic of South Korea); and antibodies to L1 were a gift from Dr. Vance Lemmon (University of Miami, Miami, FL).

Vector construction and heterologous protein expression. Sequences encoding full-length SynCAM 2 (splice product 2) (Biederer, 2006), SynCAM 3 (splice product 2), and SynCAM 4 were amplified from a mouse brain cDNA library prepared at 9–11 weeks of age (Clontech #639400). PCR products were subcloned into the eukaryotic expression vector pcMV5 for heterologous expression or for long-lasting expression in neurons into the vector pCAGGS (a gift from Dr. Jun-Ichi Miyazaki, Osaka University, Osaka, Japan) (Niwa et al., 1991).

To obtain intracellularly tagged SynCAM constructs for heterologous expression, Nef sites were introduced in full-length SynCAM sequences using the PCR mutagenesis kit (Stratagene, La Jolla, CA) to generate pcMV5 expression vectors for SynCAM1(420)*Nef, SynCAM2(391)*Nef, SynCAM3(377)*Nef, and SynCAM4(369)*Nef, with bracketed numbers indicating the amino acid into the codon of which the restriction site was introduced. A single flag epitope was inserted into the Nef site using annealed oligos. To introduce monomeric cyan fluorescent protein (CFP), its sequence was PCR amplified from pRSET-B-mCFP (a gift from Dr. Roger Tsien, University of California San Diego, San Diego, CA) and subcloned using Nhel into the pcMV5-SynCAM*Nef expression vectors. Proper sorting of tagged proteins was confirmed by surface biotinylation (data not shown). To generate SynCAM 1 and 2 constructs with an extracellular flag epitope for expression in neurons, the vectors pCAGGS-SynCAM1(420)*Nef and SynCAM2(391)*Nef were generated by PCR mutagenesis, and flag epitopes were inserted as described above. Bracketed numbers indicate the amino acid into the codon of which the restriction site was introduced.

To obtain expression vectors for the full extracellular sequences of SynCAM 2–4 fused to human IgG1, these sequences were PCR amplified and subcloned into pcMVIG9. pcMVIG9-SynCAM1 extracellular domain (ECD) has been described previously (Biederer et al., 2002). To obtain pmCMV9 expression vectors for individual or combined Ig domains of the SynCAM 1 ECD, these sequences were PCR amplified and subcloned into pCMVIG9 or pCMVIG9-SynCAM1ΔECD (Biederer et al., 2002). To express extracellular SynCAM sequences with a thrombin cleavage site N-terminal of an IgG1 sequence, sequences were amplified by PCR and subcloned into the pDT100 vector (a gift from Dr. Dimitar Nikolov, Sloan-Kettering Institute, New York, NY). To generate vectors for Semliki forest viral particle production and expression in neurons, sequences encoding wild-type, full-length SynCAM 1 and 2 were PCR amplified and subcloned into pSCE (DiGiammarino and Brenner, 1998).

For heterologous expression in human embryonic kidney-293 (HEK 293) cells, cells were transfected with FuGENE 6 (Roche Applied Science, Indianapolis, IN). COS7 cells were transfected by the DEAE-dextran method (Gorman, 1985), and IgG1 fusion proteins were purified as described previously (Sugita et al., 2001). In situ hybridization. For chromogenic detection, free-floating 40 μm sections were incubated in the appropriate riboprobe at 1 ng/μl in hybridization solution overnight at 65°C following standard procedures. Sections were then incubated in nitroblue–tetrazolium–chloride/5-
bromo-4-chlor-indolyl-phosphate (Roche Applied Science) and mounted. For fluorescence detection, 20 \( \mu \)m sections were slide-mounted and incubated with riboprobes as described for the chromogenic detection. Tissue was then incubated in block [1% blocking reagent (Roche Applied Science) in TBS and 0.1 Triton X-100 (TBST)] containing a mouse anti-NeuN antibody to detect neuronal nuclei (Mullen et al., 1992) (1:500; Millipore MAB377), followed by incubation with a polyclonal antibody against digoxigenin conjugated to alkaline phosphatase (Roche Applied Sciences) in block. Sections were rinsed, incubated in block containing anti-mouse IgG1 conjugated to Alexa 488 (1:10000; Invitro, San Diego, CA) to detect NeuN staining, then incubated in HNPP/Fast Red (Roche Applied Sciences) to detect the riboprobes, rinsed, and mounted. Riboprobe and protocol details are available on request. Digital images were collected using a Microtek (Torrance, CA) Scanmaker 9000 scanner (chromogen images) or collected using a Hamamatsu (Bridgewater, NJ) Orca camera attached to a Nikon (Tokyo, Japan) Eclipse TE2000-U microscope (fluorescence images). Signal level coding was generated using NIH ImageJ. The Lookup Table was switched to 16-color, and signal quantitation was performed using the ImageJ macro “BackgroundCorrectedDensity.” The signal for each region was then normalized to the total signal in the hippocampus and divided by the area of that region.

**Brain fractionation and glycosylation analysis.** Tissue samples were prepared from rats by rapid homogenization. Rat brain homogenates were subfractionated by the method of Jones and Matus (1974) with modifications (Biederer et al., 2002). Preparation of highly purified synaptic vesicles was performed as described previously (Takamori et al., 2006). Enzymatic glycosylation analysis was performed using sialidase (neuraminidase; Roche Applied Science) and PNGase F (N-glycosidase F; New England Biolabs, Beverly, MA) according to the manufacturer instructions. Carbohydrate contents of SynCAM extracellular domains were determined by size exclusion chromatography/laser static light scattering (SEC/LS) analysis (Hayashi et al., 1989). Analyzed proteins were expressed from pDT vectors in COS7 cells, and the IgG1 fusion protein was cleaved off with thrombin (Roche Applied Science). Amounts of purified SynCAM 4 were too low for SEC/LS analysis.

**Interaction analyses.** Bead clustering was performed using 1 \( \mu \)m diameter FluoroSpheres (Invitrogen) after covalent coupling of Protein A to the beads using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Invitrogen). SynCAM extracellular domain-IgG1 fusion proteins or control IgG were bound to Protein A-FluoSphere beads of red or green color, respectively. After agitation at 4°C for 1 h in 24-well plates, suspensions were imaged with a Hamamatsu Orca ER camera attached to a Nikon Eclipse TE2000-U. Bead clustering was not used for studies of heterophilic SynCAM proteins using Lipofectamine LTX (Invitrogen). Mature neuronal cultures were analyzed at 21 d.i.v. after immunostaining with antibodies directed against the synaptic marker synaptophysin (1:500) and the flag epitope (1:500) to visualize tagged SynCAM proteins, or against SV2 (1:500) and pleioSynCAM antibodies (T2412, 1:500) to visualize endogenous SynCAM proteins. Excitatory synaptic specializations were visualized with anti-vGlut1/2 antibodies (each 1:2000; applied in combination) and anti-PSD-95 antibodies (1:500). Inhibitory synaptic proteins shown. Synaptotagmin 1 (Syt 1) served as brain-specific control, and the widely expressed VCP and actin were additional loading controls.

**Figure 1.** SynCAM proteins are prominently expressed in brain. a. Specificity of antibodies raised for this study. HEK 293 cells were transfected with expression constructs encoding full-length mouse SynCAM 1–4. Total cell lysates were prepared, and equal lysate fractions were analyzed by immunoblotting (lanes 1–4). Twenty micrograms of adult rat forebrain proteins were analyzed as positive control (lane 5). Top to bottom, Immunoblots probed with antibodies raised against the purified, natively N-deglycosylated full-length SynCAM 1 extracellular domain (YUC8, raised in chicken), against an extracellular SynCAM 2 peptide (YUS24, raised in rabbit), against an extracellular SynCAM 3 peptide (YUS25, raised in rabbit), and against an intracellular SynCAM 4 peptide (YUS91, raised in rabbit). The numbers on the left indicate positions of molecular weight markers, and arrowheads show the running positions of the indicated SynCAM proteins. b. Tissue profile of SynCAM expression. The indicated tissues were immunostained with anti-flag antibodies by fluorescence microscopy.
Mixed coculture experiments of dissociated hippocampal neurons with HEK 293 cells expressing SynCAM proteins were performed with modifications as described previously (Biederer and Scheiffele, 2007). Briefly, HEK 293 cells expressing soluble CFP or intracellularly CFP-tagged SynCAM 1 or 2 were seeded atop neurons at 9 d.i.v. The activity analysis of extracellularly flag-tagged SynCAMs used their cotransfection with soluble CFP into HEK 293 cells to define transfected cells. Immunostaining of mixed cocultures for the presynaptic marker synapsin was performed at 11 d.i.v.; and images were acquired by confocal microscopy as described above. We used Matlab (MathWorks) to quantify the surface area fraction of HEK 293 cells that is immunopositive for synapsin. Briefly, the script uses the function [graythresh] to threshold the first channel with the fluorescence signal from a given HEK 293 cell. This defines the perimeter of the cell as mask for the second channel with the fluorescence signal of the analyzed synaptic marker. Every image was acquired with identical settings on the microscope and analyzed with the same thresholds. The output was then calculated by dividing the surface area of channel two by the surface area of the masked and thresholded channel one. The script is available on request. Images were collected blind to the synaptic marker channel.

To modify this mixed coculture assay for recruitment studies, neurons were transfected at 7 d.i.v. with a pCAGGS expression vector encoding extracellularly flag-tagged SynCAM 1 or 2 using Lipofectamine LTX. HEK 293 cells expressing CFP-tagged SynCAM 2 or 1, respectively, were seeded atop these neurons at 9 d.i.v. Immunostaining for the flag epitope and synapsin was performed at 11 d.i.v., and images were acquired by confocal microscopy as described above.

Physiological studies. To examine effects of postsynaptic SynCAM overexpression on pre-synaptic vesicle recycling, dissociated hippocampal neurons were prepared (Biederer and Scheiffele, 2007) and cotransfected at 7 d.i.v. with pCAGGS vectors encoding SynCAM proteins and soluble green fluorescent protein (GFP), or transfected with GFP alone as negative control. At 11 d.i.v., presynaptic terminals were labeled under depolarizing conditions by uptake of antibodies against the luminal domain of synaptotagmin 1 (Synaptic Systems; Cl 604.1) as described previously (Biederer and Scheiffele, 2007). After staining with appropriate secondary antibodies against synaptotagmin 1, GFP-positive neurons displaying pyramidal morphology were selected for analysis. Images of proximal dendrites were obtained using a Nikon Eclipse TE-U 2000 fluorescence microscope with an attached Hamamatsu Orca ER camera. Area and density per proximal dendrite length of synaptotagmin 1-positive puncta were determined using IPLab software (BD Biosciences Bioimaging). Analyses were performed blinded to experimental condition.

Electrophysiological analysis was performed in primary hippocampal cultures prepared from embryonic day 18 (E18) to E19 Sprague Dawley rats. Neurons were grown in Neurobasal B27 medium for 7–9 d.i.v. and

Figure 2. SynCAM proteins are predominantly expressed in neurons and display differential expression patterns in the hippocampus. a, SynCAM family members are broadly expressed in forebrain regions. Coronal sections of mouse brain at P15 were analyzed by in situ hybridization. No region was seen that lacked SynCAM staining. Scale bar, 1 mm. b, SynCAMs are expressed by neurons in the hippocampus at P15 in overlapping but distinct patterns. Immunostaining for the neuronal nuclear marker NeuN (top row) was performed in parallel with in situ hybridization for SynCAMs (third row). The merged images show that most SynCAM-expressing cells are positive for NeuN (second row; NeuN staining, red; SynCAM hybridization signal, green). Pyramidal cells of the CA fields and granule cells of the dentate gyrus differed in SynCAM expression as visualized by pseudocolor rendering of the in situ hybridization signals (bottom row). The color code shown represents signal intensities (blue, lowest signal; red, highest). SynCAM 1 displayed uniform expression across the three examined regions, whereas SynCAM 2 is more prominent in the CA1 field. SynCAM 3 and 4 appear enriched in CA1 and CA3 fields relative to dentate gyrus. Results are quantitated in c. Images correspond to typical results from four hippocampi of two mice. c, Quantification of regional SynCAM expression differences in P15 hippocampus. The pyramidal cell layers of the CA fields and granule cell layer of the dentate gyrus were outlined, and the fluorescent in situ hybridization signal in each hippocampal region was normalized to the total signal and to the area of the individual region. Bars show average SynCAM transcript hybridization signals ± SEM for four hippocampi from two animals analyzed by in situ hybridization. A.U., Arbitrary units.

specializations were detected with anti-GAD65 antibodies (1:300) and anti-gephyrin antibodies (1:500). Images were acquired on a Zeiss (Oberkochen, Germany) LSM 510 META laser scanning confocal microscope, with channels scanned separately to avoid signal contagion and a pinhole set to 1 μm for each channel. Images were quantitatively analyzed using Matlab (MathWorks, Natick, MA). Briefly, the script is based on the functions [graythresh], [bwareaopen], and [label2rgb] present in the Matlab Image Toolbox. The script is available on request.
In which brain regions are SynCAMs expressed, and do they function in neurons? The antibodies we raised were not applicable for comparative immunohistochemical detection of the four SynCAM proteins. We therefore performed in situ hybridization studies to localize SynCAM transcripts at P15 and address these questions within the peak period of synaptogenesis (Harris et al., 1992; Fiala et al., 1998). Each SynCAM is broadly expressed in forebrain, with some expression found in all brain areas examined, indicating ubiquitous functions in brain (Fig. 2a). Analysis of hippocampal sections by immunostaining for the neuronal nuclear marker NeuN combined with fluorescent in situ hybridization for SynCAMs demonstrated that all four family members are expressed predominantly in neurons at P15 (Fig. 2b). The vast majority of cells were positive both for NeuN and SynCAM transcripts, consistent with most SynCAM-expressing cells being neurons at this developmental time point. Few SynCAM-positive cells lacked NeuN staining and could be non-neuronal. We noted that neuronal expression of SynCAMs in hippocampus is not uniform. SynCAMs were differentially transcribed by the pyramidal cells of the CA fields and the granule cells of the dentate gyrus as visualized by pseudocolor rendering of the in situ hybridization signals (Fig. 2b, bottom row). Quantification of the in situ hybridization signals indicated that SynCAM 1 was evenly expressed across these hippocampal regions, whereas SynCAM 2 appeared most strongly expressed in the CA1 field (Fig. 2c). SynCAM 3 and 4 appeared enriched in both CA fields relative to the dentate gyrus. SynCAMs are therefore likely expressed in distinct ratios between different neuronal populations, contributing to the diverse surface expression patterns of neurons.

**SynCAM Ig-like domains are differentially glycosylated during brain development**

The open reading frames of the four mouse SynCAM genes encode proteins of 40.2–45.1 kDa (Biederer, 2006). However, SynCAM proteins have higher apparent molecular weights in the adult rodent forebrain (SynCAM 1, 100 kDa; SynCAM 2, 62–76 kDa; SynCAM 3, 49 kDa; SynCAM 4, 67 kDa) (Fig. 1a, lane 5). All SynCAMs contain multiple predicted N-glycosylation sites in their extracellular Ig-like domains (Biederer, 2006) that create these distinct molecular weight differences within and between SynCAM family members as shown by enzymatic deglycosylation of brain samples (Fig. 3a). Here, SynCAM 1 and 2 exist as heavily glycosylated and diverse protein species distinguished by the high amount of N-linked carbohydrates and sialic acids they carry. In contrast, SynCAM 3 and 4 carry less N-linked carbohydrates, are molecularly less diverse, and display no detectable sialic acids. After complete N-deglycosylation with PNGase F, a fraction of SynCAM 1 and all other SynCAMs are detected at the approximate molecular weights predicted by their open reading frames. O-glycosylation is not removed by these enzymatic treatments and likely accounts for the remaining SynCAM 1 population that persists at an apparent molecular weight at ~75 kDa after N-deglycosylation. Alternative splicing allows for the expression of corresponding SynCAM 1 isoforms, which can contain either none or up to 21 predicted O-glycosylation sites (Biederer, 2006).

SynCAM proteins expressed in HEK 293 cells are glycosylated similar as in adult brain (Fig. 1a). We therefore determined the carbohydrate content of their heterologously expressed extracellular domains by light scattering analysis. Consistent with our enzymatic deglycosylation results of brain SynCAMs and their apparent molecular weights, SynCAM 1 and 2 are extensively glycosylated, carrying 0.53 g of sugar/gram of protein and 0.15 g of sugar/gram of protein, respectively, whereas SynCAM 3 contains only 0.04 g of sugar/gram of protein. This establishes the
four SynCAM proteins as complex glycoproteins primarily expressed in brain.

To guide our understanding of SynCAM function in brain development, we determined their protein expression profile in the rat brain (Fig. 3b). SynCAM 1 and 4 are already expressed in late embryonic stages, whereas SynCAM 2 and 3 are first expressed postnatally around P3. All SynCAMs are prominently expressed by the second week after birth. Their postnatal expression profile parallels the period of intense synapse formation that occurs between days 6 and 15 (Harris et al., 1992; Fiala et al., 1998). The four SynCAM proteins continue to be expressed into adulthood, albeit at lower levels (Fig. 3b) (data not shown). At P15, additional N-glycosylated SynCAM 1 species of apparent 70–90 kDa molecular weight are prominent and appear together with its 100 kDa species present throughout development and into adulthood. SynCAM 3 also undergoes a developmentally regulated molecular weight shift at P15, dropping in its apparent molecular weight from 58 to 49 kDa. These results demonstrate that SynCAM proteins are coexpressed in the developing brain and therefore have the potential to share functions with SynCAM 1 during synaptogenesis and can be modified with carbohydrates in a developmentally regulated manner.

SynCAMs are synaptic plasma membrane proteins

SynCAMs are synaptic plasma membrane proteins present at excitatory but also found at inhibitory synaptic specializations. a, Synaptic plasma membrane fractionation of SynCAM proteins. The indicated subcellular fractions were prepared from rat forebrain at P9. Thirty micrograms of each fraction were analyzed by immunoblotting for the four SynCAM proteins as indicated. The synaptic membrane protein N-cadherin, the synaptic vesicle protein synaptophysin, and the soluble protein GDI were markers for these respective fractions. The numbers on the left indicate positions of molecular weight markers. Arrowheads show the running positions of the indicated SynCAM proteins in their predominantly adult (black) and postnatal (white) glycosylation forms. b, Exogenously expressed SynCAM 1 is sorted to both excitatory and inhibitory synaptic specializations in dissociated hippocampal neurons. Epitope-tagged SynCAM 1-flag was transfected into dissociated hippocampal neurons at 7 d.i.v. After transfection, mature cultures were analyzed at 21 d.i.v. by confocal microscopy after triple immunostaining for flag, the inhibitory presynaptic vesicle marker GAD65, and the postsynaptic excitatory marker PSD-95. Individual immunostainings are shown in the gray scale panels as indicated. The two panels at the bottom depict the indicated double merged images (SynCAM 1-flag, red; PSD-95, green; GAD65, blue). Insets in the top right of each panel represent 10-fold enlarged areas of the image shown. Confocal images were analyzed using Matlab for the number of single puncta and the occurrence of their double colocalization (SynCAM 1-flag, 2461 puncta analyzed; 3 images). Double colocalization identifies 53 ± 23% of SynCAM 1-flag puncta as immunopositive for PSD-95, and 24 ± 12% of SynCAM 1-flag puncta as immunopositive for GAD65. Errors are stated as SDs. The remaining SynCAM 1-flag puncta are removed from the synaptic markers analyzed here and nonsynaptic. We observed an occasional colocalization of GAD65 and PSD-95 in our hippocampal cultures, quantitated as an — 10% mismatch of excitatory and inhibitory synaptic specializations similarly as described previously (Anderson et al., 2004). Scale bar, 10 μm. c, SynCAM 2 colocalizes with excitatory and inhibitory synapse markers in cultured hippocampal neurons. Epitope-tagged SynCAM 2-flag was expressed and analyzed as described in a (SynCAM 2-flag, 1951 puncta analyzed; 4 images). Double colocalization identifies 62 ± 28% of SynCAM 2-flag puncta as immunopositive for PSD-95, and 31 ± 12% of SynCAM 2-flag puncta as immunopositive for GAD65. Scale bar, 10 μm. The immunostainings shown in b and c are representative of the quantification results.

We complemented this biochemical analysis by studying SynCAM localization in cultured neurons. In the absence of antibodies applicable for immunostaining, we expressed epitope-tagged constructs in dissociated hippocampal neurons at 7 d.i.v., when neurons in culture can rapidly form synapses (Fletcher et al., 1994). In these constructs, one extracellular flag epitope was inserted C-terminal of the third Ig-like domain to minimally interfere with putative intracellular sorting signals. In transfected neurons, SynCAM 1-flag exhibited a mostly punctate staining...
pattern with some of the signal being diffuse (Fig. 4b), similar to other synaptic membrane proteins such as syntaxin (Garcia et al., 1995). To gain better insight into the distribution of SynCAM proteins in neurons, we performed triple immunostainings of SynCAM 1-flg with the inhibitory presynaptic marker GAD65 and the excitatory presynaptic marker PSD-95 (Fig. 4b). Quantification showed that SynCAM 1-flg was sorted to both types of synaptic specializations but was preferentially detected at PSD-95-labeled excitatory sites, with which ~53% of SynCAM 1-flg puncta colocalized, compared with 24% of its puncta being immunopositive for GAD65. Similarly, SynCAM 2-flg exhibited a punctate pattern in hippocampal neurons and was detected at both excitatory and inhibitory sites, with ~62% of SynCAM 2-flg in colocalization with PSD-95 and 31% with GAD65 (Fig. 4c). This analysis is consistent with our observation that both excitatory and inhibitory synaptic specializations are immunopositive for endogenous SynCAMs 1, 2, and 3, which were detected by our pleioSynCAM antibody (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

To confirm that exogenously expressed SynCAM proteins indeed are sorted to synaptic sites as defined by aligned presynaptic and postsynaptic specializations, we performed triple colocalization of SynCAM 1-flg or SynCAM 2-flg with the presynaptic marker SV2 and the postsynaptic marker PSD-95 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Approximately 45% of detected SynCAM 1-flg and 67% of SynCAM 2-flg puncta colocalized with both markers, confirming their presence at aligned synapses (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). These results are consistent with the primarily synaptic localization of endogenous SynCAMs 1, 2, and 3 (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Together, our results demonstrate that SynCAM proteins are synapse components present at excitatory as well as inhibitory synaptic specializations.

Homophilic cell adhesion is conveyed by SynCAMs 1, 2, and 3

The fact that SynCAMs are expressed in different regional patterns (Fig. 2b,c) suggested that they may confer distinct adhesive properties to neuronal populations differentially expressing SynCAM proteins. To identify these properties, we first addressed whether all SynCAM proteins mediate homophilic cell adhesion similar to SynCAM 1. Using fluorophore-labeled beads coated with each SynCAM extracellular domain, we determined whether these domains conferred bead clustering, indicative of binding to themselves (Fig. 5a). The extracellular domains of SynCAM 1, 2, and 3 caused extensive clustering of beads, consistent with previous reports for SynCAM 1 and 3 (Biederer et al., 2002; Kakunaga et al., 2005). This demonstrates that each of these three proteins, including the previously uncharacterized SynCAM 2, can engage in a homophilic interaction. In contrast, beads coated with the SynCAM 4 extracellular domain did not adhere to each other, excluding strong interactions of this protein with itself under this condition. The same SynCAM 4 extracellular domain protein conferred heterophilic binding (see below) (Fig. 6a), showing that it is properly folded and capable of engaging in its correct adhesive interactions.

To confirm that SynCAM extracellular domains engage themselves in cell adhesive trans-interactions, we individually expressed full-length SynCAMs in HEK 293 cells. The expressed proteins were tagged with CFP within their cytosolic sequence for protein detection. Cells were analyzed by fluorescence microscopy for homophilic trans-interactions of SynCAM proteins at sites of cell–cell contact (Fig. 5b). In agreement with the bead clustering assay, we observed cell adhesive interactions of SynCAMs 1, 2, and 3. Their homophilic interaction recruited almost all detectable SynCAM proteins from the surrounding plasma membrane to sites of cell–cell contact, where they formed extended zipper-like structures. Homophilic cell adhesion was not detected for SynCAM 4, consistent with the bead clustering assay results. These results demonstrate that SynCAM 1, 2, and 3, but not 4, are strong homophilic cell adhesion molecules and identify that SynCAMs can exhibit distinct extracellular interactions. They therefore have the potential to mediate unique functions in neuronal adhesion.

Strong and specific heterophilic interactions define each SynCAM family member

Our observations that SynCAM proteins can exert distinct extracellular interactions and are differentially expressed prompted us to examine their adhesive properties in biochemical detail. Based on our analysis of sequence conservation in the SynCAM Ig-like domains (Biederer, 2006), we hypothesized that different Syn-
CAMs might bind each other. To address this question, we first performed affinity chromatography experiments and loaded detergent extracts from membrane preparations of rat forebrain on beads containing equal amounts of immobilized extracellular domains of each individual SynCAM family member expressed as IgG-fusion proteins (Fig. 6a). SynCAM 1 showed weak, salt-sensitive homophilic binding (data not shown) (asterisks in Fig. 6a indicate cross-reactive bands). In contrast, SynCAM 1 was retained strongly in a primarily salt-resistant manner on the SynCAM 2 extracellular domain (Fig. 6a, first row, lanes 7, 8). No binding of SynCAM 1 to other family members was detected. Reciprocally, SynCAM 2 bound strongly to the SynCAM 1 extracellular domain (Fig. 6a, second row, lanes 3, 4) but not to any other SynCAM protein. SynCAM 3 engaged in a strong interaction with SynCAM 4 (Fig. 6a, third row, lanes 16, 17) in agreement with a recent study (Maurel et al., 2007; Spiegel et al., 2007). The complementary binding of brain SynCAM 4 to immobilized SynCAM 3 was not observed in this assay, possibly because of an excess of SynCAM 3 over SynCAM 4 in the brain homogenate used as starting material for binding (data not shown), which could lead to sequestration of free SynCAM 4 in the detergent extract. SynCAM 3 additionally showed weak binding to SynCAM 1 (Fig. 6a, third row, lanes 3, 4). The observed heterophilic SynCAM 1/2 and 3/4 interactions were primarily resistant to a high 800 mM salt washing step, indicative of their high strength and specificity. We could not detect the homophilic interactions of SynCAM 2 or 3 in this assay, possibly because affinity chromatography is a rather stringent assay of adhesive properties (Boucard et al., 2005), or because of the need for a specific interaction geometry that may be impaired in this experimental approach. Importantly, our results underline that the prominent binding of SynCAM 2 to SynCAM 1 detected by affinity chromatography appears stronger than the homophilic interaction of either of them alone. Similarly, retention of SynCAM 3 on SynCAM 4 is readily detected in contrast to SynCAM 3 homophilic binding, indicating that SynCAM 3 also prefers heterophilic interactions.

Next, we analyzed SynCAM 1 functions as a heterophilic cell adhesion molecule using full-length constructs tagged with different intracellular epitopes (Fig. 6b–d). HEK 293 cells expressing flag-tagged SynCAM proteins were mixed with cells expressing different CFP-tagged SynCAM family members. Contact sites between cells expressing different SynCAM proteins were analyzed by fluorescence microscopy. SynCAM 1 engaged SynCAM 2 in a heterophilic cell adhesion interaction (Fig. 6b), consistent with our affinity chromatographic analysis. The intercellular interaction of SynCAM 1 and 2 caused zipper-like structures to form at cell contact sites, in agreement with strong heterophilic adhesion of these two proteins. The weak binding of SynCAM 3 to SynCAM 1 detected biochemically (Fig. 6a) did not mediate cell adhesion under these conditions (Fig. 6c). Additionally, the heterophilic adhesion of SynCAM 3 and 4 was observed as zipper- or grid-like structures (Fig. 6d). As expected, no adhesive interaction of SynCAM 1 with 4 was detected (data not shown). SynCAM 1/2 and SynCAM 3/4 therefore constitute specific, cognate pairs of heterophilic neuronal adhesion molecules. Together, our analysis defines that SynCAM proteins engage each other in highly specific interactions that distinguish them individually, reminiscent of an adhesive code (Fig. 6e).

SynCAM adhesion is mediated by the first two Ig-like domains and is regulated by glycosylation

To better characterize SynCAM adhesion complexes, we aimed to identify the domains that mediate the heterophilic interactions of SynCAM 1 (Fig. 7a). We immobilized IgG-fusion proteins corresponding to the three individual or combined Ig-like domains of SynCAM 1 and incubated them with detergent extracts from membrane preparations of rat forebrain. The extracellular partner SynCAM 2 was retained by the combined first and second Ig-like domains of SynCAM 1 as efficiently as by the full SynCAM 1 extracellular domain (Fig. 7a, lanes 8 vs 4). The combined second and third Ig-like domains of SynCAM 1, however, did not bind SynCAM 2 (lane 13). Individually, only the first Ig-like domain of SynCAM 1 exerted weak interactions with SynCAM 2 (lane 17), whereas the second and third Ig-like domains alone showed no retention, identical to negative control beads (data not shown). These results indicate that the first two Ig-like domains of SynCAMs together mediate efficient trans-interactions as depicted in Figure 6e. Notably, one individual lg domain is ~4.0 nm long (Davies et al., 1975). Assuming that two SynCAM extracellular domains engage each other in an extended conformation, the overall length of their complex would be approximately equivalent to four Ig-like domains in tandem flanked by the sequences at the stalk of their extracellular domains. For human SynCAM 1 and 2, these flanking sequences are 31–70 and 18–58 aa long, respectively, depending on alternative splicing of this region (Biederer, 2006). Assuming an α-helical conformation of these stalks, the extracellular portion of a fully extended SynCAM 1/2 complex can be predicted to have a length of 23–35 nm.

The first two Ig-like domains of SynCAM 1 contain four predicted N-glycosylation sites (Biederer, 2006). Considering that SynCAM 1 undergoes developmentally regulated N-glycosylation, we asked whether this modification of its Ig-like domains may affect its extracellular interactions. We performed affinity chromatographies of solubilized rat membrane proteins on the natively N-deglycosylated SynCAM 1 extracellular domain or on the control-treated extracellular domain (Fig. 7b). Enzymatic removal of N-linked carbohydrates on SynCAM 1 reduced its strong retention of SynCAM 2 and its weaker binding to SynCAM 3 each approximately threefold to fourfold. The extensive modification of SynCAM 1 with N-linked carbohydrates therefore promotes its extracellular interactions, possibly by providing for a structural organization of the extracellular domains that fits the conformation they adopt during adhesion. Developmental changes in SynCAM 1 glycosylation therefore may serve to regulate its adhesive interactions.

SynCAM 1 and 2 assemble into an adhesive synaptic complex in vivo

Our localization and binding studies performed in vitro indicated that these heterophilic SynCAM interactions could occur at synaptic membranes in vivo. The strong heterophilic binding observed between SynCAM 1 and 2 was of particular interest because SynCAM 2 may mediate synaptic effects of SynCAM 1 (Biederer et al., 2002; Sara et al., 2005). To test whether these two adhesion molecules interact at synapses, we performed coimmunoprecipitation experiments (Fig. 8). Synaptosomes were prepared from rat brains at P15, when all SynCAM proteins are prominently expressed. Synaptosomal membrane proteins were solubilized and subjected to immunoprecipitation with a specific monoclonal antibody against the SynCAM 1 extracellular sequence. Immunoprecipitates were enriched for SynCAM 1 (Fig. 8, top, lane 3), whereas control antibodies did not precipitate SynCAM 1 or any other protein analyzed (lane 5). Importantly, SynCAM 1 antibodies coimmunoprecipitated SynCAM 2 from synaptosomal membranes (Fig. 8, second panel, lane 3) but none
Specific heterophilic interactions define cognate SynCAMs to mediate cell adhesion. 

**a.** Analysis of SynCAM interactions by affinity chromatography. Membrane proteins from adult rat forebrains were solubilized to obtain a detergent extract (lanes 1, 10, 19) and loaded on beads containing covalently immobilized extracellular domains of SynCAM 1 (lanes 2–4), SynCAM 2 (lanes 6–8), SynCAM 3 (lanes 11–13), SynCAM 4 (lanes 15–17), or a control Ig protein (lanes 20–22). The flow-through (FT) of each affinity chromatography was obtained, beads were washed, subsequently eluted with buffer containing high salt at 800 mM, and finally eluted with SDS. Samples were analyzed by immunoblotting for each of the four SynCAM proteins as shown, using monoclonal chicken antibodies to detect SynCAM 1, and with an antibody detecting the NGL family of synaptic membrane proteins as negative control. To control for cross-reactivity of anti-SynCAM antibodies with SynCAM extracellular domains used as affinity matrix that were washed off the beads and were present in the eluate samples, beads containing only the purified extracellular domains were eluted with SDS, and an eluate amount equal to the respective affinity chromatography fraction was loaded (lanes 5, 9, 14, 18). Antibody cross-reactive bands are marked by asterisks. Strong heterophilic

**Figure 6.** Specific heterophilic interactions define cognate SynCAMs to mediate cell adhesion.
of the other SynCAMs or control proteins. To define whether these interactions are altered during brain development, we performed these coimmunoprecipitation experiments further in adult rat brain and determined that SynCAM 1 and 2 formed a physical complex in adult brain to an apparently identical extent as at P15 (data not shown). Notably, also in adult brain, no interactions of SynCAM 1 and 3 were observed. This coimmunoprecipitation of SynCAM 2 with SynCAM 1 is consistent with our affinity chromatography results and extends our analysis of SynCAM complex formation to native conditions in brain. The heterophilic interaction of SynCAM 1 and 2 is therefore specific and occurs throughout development in synaptosomal membranes in vivo.

**SynCAM 1 and 2 recruit presynaptic markers**

Do SynCAM 1 and SynCAM 2 have comparable functions at synapses? To begin to address this question, we performed mixed cocultures of hippocampal neurons with HEK 293 cells expressing either of these two proteins on their surface. To visualize transfected HEK 293 cells, we used the CFP-tagged SynCAM 1 and 2 constructs that had been used for the cell adhesion assays shown in Figure 5b. The activity of SynCAMs to organize presynaptic specializations was quantitatively assessed by determining the surface area of HEK 293 cells that was immunopositive for recruited presynaptic vesicle protein synapsin in coculture with neurons (Fig. 9a). SynCAM 1 expression in cocultured HEK 293 cells caused a significant increase of synapsin-positive puncta atop the cell surface compared with negative control HEK 293 cells expressing soluble CFP alone (Fig. 9b) as described previously (Biederer et al., 2002). Importantly, expression of SynCAM 2 similarly resulted in the significant detection of synapsin-positive puncta atop HEK 293 cells. These results indicate that both SynCAM 1 and 2 organize presynaptic specializations in this mixed coculture assay. We also analyzed the extracellularly flag-tagged SynCAM 1 and 2 proteins in this assay and observed that their activity was indistinguishable from the described SynCAM constructs with native extracellular sequences (data not shown). This demonstrates that they are functional and suitable for the localization studies shown in Figure 4, b and c, and below.

**SynCAM 1 and 2 recruit each other into adhesive neuronal complexes**

Do the heterophilic interactions of SynCAM 1 and 2 only occur at stable synapses or can they recruit SynCAMs to sites of contacts with differentiating neurons? We analyzed this question in a modified coculture system of dissociated hippocampal neurons...
expressing extracellularly flag-tagged SynCAM 2 with HEK 293 cells expressing CFP-tagged SynCAM 1 (Fig. 9c). After 2 d of coculture, neuronal SynCAM 2-flag was strongly recruited to sites of contact with SynCAM 1 expressed in HEK 293 cells (Fig. 9c). Serial reconstruction of confocal images demonstrated that the SynCAM 2-expressing neurons formed extensive membrane contacts containing concentrated amounts of neuronal SynCAM 2-flag that enveloped SynCAM 1 expressing HEK 293 cells (Fig. 9c, second and third rows). Immunostaining for the presynaptic marker synapsin atop the HEK 293 cells expressing SynCAM 1 served as positive control for its activity to organize presynaptic specializations in these coculture experiments (Fig. 9c, fourth row). The staining of HEK 293-expressed SynCAM 1 does not extensively mirror the staining of presynaptic SynCAM 2-flag in the contacting neuron, showing areas lacking overlap with neuronal SynCAM 2-flag. This can be expected because of the assay design, with multiple neurons in addition to the one transfected neuron contacting a given HEK cell that all can contribute neuronal interaction partners with HEK cell-expressed SynCAM 1. Together, these results reveal that the adhesive SynCAM code is instructive for the specific assembly of the SynCAM 1/2 complex across membranes.

To better understand the dynamics of synaptic SynCAM assembly, we next tested whether SynCAM 1 and 2 can also operate in reverse. Following the same paradigm as outlined above, we analyzed hippocampal neurons expressing SynCAM 1-flag that were cocultured with HEK 293 cells expressing CFP-tagged SynCAM 2 for SynCAM recruitment (Fig. 9d). SynCAM 2 presented from the HEK 293 cell surface was able to assemble into a complex with neuronal SynCAM 1-flag. In conjunction, HEK cell-expressed SynCAM 2 mediated synapsin recruitment from the surrounding neurons. Specificity of SynCAM 1/2 assembly was confirmed by the inability of negative control HEK 293 cells expressing CFP alone to assemble neuronal SynCAM 1 complexes after surface contact, in parallel to the lack of synapsin clusters atop these control HEK 293 cells (Fig. 9e). Together, we demonstrate that the interactions of SynCAM 1 and 2 on neuronal surfaces are reciprocal and recruit each other into adhesive complexes.

SynCAM 1 and 2 promote functional synapses

Based on our analysis of SynCAMs in cocultures, we aimed to understand whether SynCAM 1 and 2 also operate between neurons to organize synapses and control synaptic function. To address these questions, we overexpressed SynCAM 1 or 2 in dissociated hippocampal neurons. First, we determined whether SynCAM 1 and 2 alter the density of functional presynaptic terminals formed between neurons in culture (Fig. 10a, b). Hippocampal neurons were transfected at the beginning of the peak period of synaptogenesis in culture at 7 d.i.v. with vectors encoding wild-type SynCAM proteins and soluble GFP. We used a low-efficiency transfection method to increase SynCAM expression only in a small subset of neurons and, hence, to elevate SynCAM protein amounts only in one of the cells that is in contact with other neurons. Four days later, at the height of endogenous synaptogenesis, we briefly depolarized the neurons and labeled all active presynaptic specializations by uptake of antibodies directed against the luminal domain of synaptotagmin 1 into recycling synaptic vesicles (Matteoli et al., 1992; Biederer and Scheiffele, 2007). Analysis of the density of puncta positive for synaptotagmin 1 uptake was performed along the dendrites of SynCAM-overexpressing pyramidal neurons identified through their GFP signal (Fig. 10a). This allowed us to directly determine the number of active presynaptic terminals formed atop neurons with elevated SynCAM expression (Fig. 10b). Neurons expressing only GFP served as negative control. Postsynaptic SynCAM 2 overexpression resulted in a significant increase by 34 ± 9% in the density of presynaptic terminals containing recycling synaptic vesicles along neuronal dendrites. SynCAM 1 overexpression caused a trend to increased presynaptic terminal density, which was not statistically significant. Our results show that postsynaptic SynCAM 1 and 2 engage incoming axons in transsynaptic interactions, which in turn causes contacting presynaptic neurons to display more terminals.

To determine whether these new presynaptic terminals organized by SynCAMs are functional, we transduced hippocampal neurons at 6–8 d.i.v. with expression vectors encoding full-length SynCAM 1 or 2 proteins and soluble GFP. Miniature EPSCs (mEPSCs) were recorded from GFP-expressing neurons 12–24 h later (Fig. 10c, d). SynCAM 1 overexpression increased mEPSC frequency when compared with untransfected control neurons in the same culture (Fig. 10d) or to GFP-transfected neurons (data not shown) as reported previously (Biederer et al.,

![Figure 8. SynCAM 1 and 2 form a specific adhesive complex in synaptic membranes in vivo. Synaptosomes were prepared from rat forebrain at P15 during the peak of SynCAM expression and synaptogenesis. Synaptosomal membrane proteins were extracted with detergent (lane 1) and immunoprecipitated with specific antibodies against the extracellular domain of SynCAM 1 (lanes 2, 3) or control antibodies (lanes 4, 5). The flow-through (FT) of each immunoprecipitation was obtained, and immunoprecipitates (IP) were collected on beads, washed, and eluted with SDS. The same amount of antibody used for the immunoprecipitation was loaded to identify cross-reactive bands recognized by secondary antibodies (lane 6), which are marked by asterisks. Fractions were probed for each of the four SynCAM proteins as indicated, and with an antibody detecting the L1 Ig superfamily member and the synaptic vesicle protein synaptophysin as negative control membrane proteins. SynCAM 1 coinmunoprecipitated SynCAM 2 from synaptosomes but no other SynCAM family member (lane 3). Numbers on the left indicate positions of molecular weight markers. Input and FT lanes contain 4% of the extract amount used for each immunoprecipitation.](image-url)
heterophilic adhesion pattern. In drawing SynCAM proteins is their highly distinct
ration or stabilization. M.R.A., and T.B., unpublished results),
pressed throughout adulthood (A.I.F.,
roligins and other synaptic proteins (Song
crease in brain expression typical for neu-
ally, they show the gradual developmental
stion complexes exist, and that SynCAM
proteins participate in synaptic organiza-
tion and contribute to synaptic function.

The properties of SynCAMs described
here are consistent with their transsynaptic
localization and point to a common synaptic
function. In the hippocampus, all Syn-
CAMs are expressed mostly by neurons
during the peak period of synaptogenesis
around the second postnatal week. Syn-
CAM 1 and 2 are present at synapses, and
the trans-interaction of their first two Ig-
like domains suggests an extended Syn-
cam complex that would fit the 20 nm
cleft width of central synapses (Schikorski
and Stevens, 1997; Ahmari and Smith,
2002). SynCAMs are ubiquitously ex-
pressed throughout the brain, indicating a
wide-ranging function, but also display no-
table differences in their regional profiles,
consistent with nonoverlapping roles in
distinct neuronal populations. Addition-
ally, they show the gradual developmental
increase in brain expression typical for neu-
roligins and other synaptic proteins (Song
et al., 1999). SynCAM proteins remain
expressed throughout adulthood (A.L.F.,
M.R.A., and T.B., unpublished results),
when they may function in synapse matu-
ration or stabilization.

An intriguing biochemical property of
SynCAM proteins is their highly distinct
heterophilic adhesion pattern. In drawing

Discussion
Our results reveal SynCAMs as synaptic
membrane proteins that assemble into spe-
cific adhesion complexes, with the cognate
partners SynCAM 1 and 2 recruiting each
other to neuronal membrane sites. Nota-
bly, both SynCAM 1 and 2 can organize
synapses and promote excitatory synaptic
transmission. Together, we demonstrate
that molecularly defined SynCAM adhe-
sion complexes exist, and that SynCAM
proteins participate in synaptic organiza-
tion and contribute to synaptic function.

Together, our results demonstrate that
SynCAM proteins bind each other in specific
heterophilic patterns and identify SynCAM 1
and 2 as components of cognate transsynap-
tic complexes. Concurringly, the com-
ponents of the SynCAM 1/2 complex each orga-
nize functional synapses and potentiate
excitatory synaptic transmission.

Figure 9. SynCAM 1 and 2 recruit presynaptic marker proteins and engage each other in adhesive neuronal complexes. a,
SynCAM 1 and 2 recruit synapsin in a mixed coculture assay. HEK 293 cells expressing CFP-tagged SynCAM 1 or CFP-tagged
SynCAM 2 were seeded atop dissociated hippocampal cultures at 9 d.i.v. HEK 293 cells expressing soluble CFP alone served as
negative control. Cocultures were analyzed at 11 d.i.v. by confocal microscopy for localization of the presynaptic vesicle marker
synapsin (red) and CFP (green). Merged stacks of serial optical sections are shown. Synapsin puncta were detected atop SynCAM
1- and SynCAM 2-expressing HEK 293 cells and are marked with white arrowheads in the images shown. b, Expression of SynCAM
1 or SynCAM 2 in HEK 293 cells cocultured with hippocampal neurons caused a significant increase of synapsin-positive puncta
covering the cell surface area compared with control HEK 293 cells expressing CFP alone. The activity of both SynCAMs was similar
(CFP alone, 4.9 ± 1.0% synapsin area coverage, n = 15 cells; SynCAM 1-CFP, 10.7 ± 1.9%, p = 0.011 relative to CFP, n = 15
cells; SynCAM 2-CFP, 16.5 ± 3.2%, p = 0.005 relative to CFP, n = 20 cells). Statistical analyses were performed using unpaired
t tests with two-tailed p values. c, SynCAM 1 recruits and retains neuronal SynCAM 2 in adhesive neuronal complexes. Dissociated
hippocampal neurons were transfected at 7 d.i.v. with flag-tagged SynCAM 2 to allow its visualization by immunostaining. HEK
293 cells expressing CFP-tagged SynCAM 1 and 2 were seeded atop dissociated hippocampal cultures at 9 d.i.v., and cocultures were
analyzed at 11 d.i.v. by confocal microscopy for localization of both SynCAM proteins and the presynaptic vesicle marker synap-
sin. Optical sections of each 1 μm thickness were obtained. The top panel shows, for a single optical section, the merged image
(SynCAM 2-flag, red; SynCAM 1-CFP, green; synapsin, blue). The four panels at the bottom show for two serial optical sections the
indicated individual immunostainings in grayscale and the triple merged image in color. White arrowheads mark synapsin
positive puncta formed atop the SynCAM 1-expressing HEK 293 cell. d, SynCAM 2 reciprocally recruits neuronal SynCAM 1 into
adhesive complexes. Analysis was performed as described in c in cocultures of hippocampal neurons expressing flag-tagged SynCAM 1 and
HEK 293 cells expressing CFP-tagged SynCAM 2. The top panel shows, for a single optical section, the merged staining of the three
indicated proteins (SynCAM 1-flag, red; SynCAM 2-CFP, green; synapsin, blue). The panels below show two serial optical sections
obtained after immunostaining for the indicated proteins as described in c. e, Control HEK 293 cells do not retain SynCAM
proteins. Analysis was performed as described in c in cocultures of hippocampal neurons expressing flag-tagged SynCAM 1 and
HEK 293 cells expressing CFP alone. After surface contact of neurons expressing SynCAM 1, no synapsin retention or SynCAM
complex formation was observed atop HEK 293 cells.
domains promotes its binding to SynCAM. The heterophilic interactions between SynCAM family members 1/2 and 3/4 are therefore likely to define each SynCAM functionally in neurons and are a striking property reminiscent of a neuronal adhesive code. Furthermore, SynCAM 1 and 2 drive the recruitment of each other to sites of neuronal contact, demonstrating that their transsynaptic interaction can occur in differentiating neurons. The heterophilic binding of SynCAM 3 and 4 shown here was also reported recently in a study of myelination in the peripheral nervous system (Maurel et al., 2007; Spiegel et al., 2007). This heterophilic specificity distinguishes SynCAMs from the neurexin-neurologin system, which is mainly controlled by alternative splicing along with certain isoform preferences (Ichthchenko et al., 1996; Boucard et al., 2005; Chih et al., 2006; Comolli et al., 2006; Graf et al., 2006). SynCAM sequences and domain organization are highly conserved within this protein family (Biederer, 2006), and the molecular basis for the specificity of SynCAM binding is not yet understood because structural information is lacking. However, it is of interest that SynCAMs are expressed as complex glycoproteins, with SynCAM 1 and 3 undergoing a developmentally regulated glycosylation that is unique among synaptic adhesion molecules. The fact that N-glycosylation of the SynCAM 1 Ig-like domains promotes its binding to SynCAM 2 points to a role of this modification in regulating the strength and specificity of SynCAM adhesion. The mechanisms providing for such differential SynCAM glycosylation in a developmentally regulated manner are presently unknown.

Both SynCAM 1 and 2 are highly expressed in the hippocampus (M.R.A. and T.B., unpublished results), indicating their functional relevance in this region. This motivated us to determine their roles in hippocampal neurons and synapses. In addition, the analysis of synaptic functions of SynCAM 1 in hippocampal neurons (Biederer et al., 2002; Sara et al., 2005) provided a framework for our studies. We investigated synaptic effects by overexpressing individual SynCAM proteins to overcome the problems that loss-of-function approaches pose because of redundancy between parallel synaptic adhesions systems. Three results now identify that the heterophilic adhesion molecules SynCAM 1 and SynCAM 2 can organize synapses and contribute to their function. First, both SynCAM 1 and SynCAM 2 expressed in HEK 293 cells recruit presynaptic marker proteins to contact sites with hippocampal neurons. Second, elevated SynCAM 2 in dendritic membranes increases the density of functional presynaptic terminals atop these dendrites as observed by visualizing recycling synaptic vesicles. Third, overexpression of either SynCAM 1 or SynCAM 2 in neurons increases mEPSC frequency, consistent with SynCAMs promoting functional excitatory synaptic transmission. It remains presently unclear how SynCAM 1 promotes mEPSCs when exogenously expressed in hippocampal neurons without significantly increasing the number of presynaptic terminals between neurons. This could be caused by effects on the presynaptic release machinery in contacting neurons, or because of a conceivable conversion of existing silent to functional synapses by postsynaptic SynCAM 1. The fact that SynCAM 1 expression in postsynaptic neurons only provides for a trend toward more presynaptic specializations, whereas it causes the significant recruitment of presynaptic markers when expressed in HEK 293 cells, is possibly a result of the high surface expression of heterologous cells and their lack of the endogenous SynCAM amounts found in neurons, providing for a more pronounced readout in the mixed coculture assays.

In comparing SynCAM activities, we noted that increasing SynCAM 2 amounts appeared more effective than elevating SynCAM 1. It is interesting to speculate that SynCAM 2 availability may set a threshold that determines physiological effects of SynCAM-mediated synaptic organization. Consistent with this
hypothesis, SynCAM 1 appears somewhat less synaptic than SynCAM 2, indicating that it may be constitutively available on neurite membranes for possible recruitment by SynCAM 2 to organize nascent synaptic sites and potentially align them. This hypothesized role of SynCAM 1 would be analogous to nonsynaptic neuroligin 1 providing hotspots of synapse formation on dendrites (Gerrow et al., 2006). However, such analyses of SynCAM complex assembly will have to await the ultrastructural subsynaptic localization of each SynCAM family member, and determining the extent of their presynaptic versus postsynaptic localization will provide insight into the structure and function of SynCAM complexes. Although SynCAMs are found at both presynaptic and postsynaptic membranes (Biederer et al., 2002), lateral cis-interactions may occur in addition to the binding of SynCAMs in trans-interactions, which could serve to regulate their transsynaptic interactions similar as described previously for neurexins and neuroligins (Taniguchi et al., 2007) and for cadherin-mediated adhesion (Tanaka et al., 2000). With respect to transsynaptic functions of SynCAM complexes, it is notable that neurexins also are found at both presynaptic and postsynaptic membranes (Taniguchi et al., 2007). Because the protein interaction motifs in the cytosolic SynCAM and neurexin sequences are highly conserved (our unpublished observations), the SynCAM and neurexin/neuroligin adhesion systems may therefore engage similar intracellular partners on both sides of the synaptic cleft to organize synapses.

Together, we identify the four SynCAM proteins as a family of neuronal adhesion molecules defined by highly specific heterophilic interactions and characterize the SynCAM 1/2 complex as an asymmetric synaptic adhesion system regulated by N-glycosylation. We demonstrate that SynCAM 1 and 2 proteins are localized to synapses, act across membranes to organize synaptic sites, and promote synaptic transmission. This study therefore introduces SynCAMs as components of a novel heterophilic transsynaptic system. How do SynCAM molecules cooperate with other transsynaptic interactions at the large variety of synapses found in the CNS? It is intriguing to hypothesize a balance between adhesion systems such as SynCAMs that could guide synaptic development and organization, those that affect both synapse organization and excitatory/inhibitory specificity, such as neurexins and neuroligins, as well as others that promote synapse maturation such as N-cadherins. These transsynaptic interactions are likely rather complex considering the additional roles of postsynaptic SALM (synaptic cell adhesion-like molecule) and NGL proteins and EphB receptors in neuronal and synaptic differentiation (Kays et al., 2006; Kim et al., 2006; Ko et al., 2006; Wang et al., 2006) and may ultimately not only differentiate but also specify maturing synapses (Benson et al., 2001). On this network level, SynCAM expression differences between neuronal populations combined with their precise heterophilic interaction patterns could contribute to the specificity of synaptic connectivity in addition to organizing individual synapses.

References


Fogel et al. • Synaptic Adhesion by SynCAM Complexes


Supplemental Data

SynCAMs Organize Synapses Through Heterophilic Adhesion

Abbreviated Title: Synaptic Adhesion by SynCAM Complexes

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Characterization of SynCAM antibodies.
HEK 293 cells were transfected with expression constructs encoding full-length mouse SynCAM 1-4 and equal lysate fractions were analyzed by immunoblotting (lanes 1-4). 20 µg adult rat forebrain proteins were analyzed as positive control (lane 5). The top immunoblot was probed with a commercially available monoclonal antibody against SynCAM 1, showing that it is isoform specific (MBL Laboratories clone 3E1, raised in chicken). The bottom immunoblot was probed with a polyclonal antibody raised against the intracellular extreme C-terminus of SynCAM 1 (T2412, raised in rabbit) (Biederer et al., 2002) that detects SynCAMs 1, 2 and 3 to a comparable extent and serves as a pleioSynCAM antibody. Numbers on the left indicate positions of molecular weight markers and arrowheads show the running positions of the indicated SynCAM proteins. IB, immunoblotting.

Supplemental Figure 2. Subsynaptic fractionation shows that SynCAM 3, but not other SynCAM proteins, are present in synaptic vesicles.
Brain homogenate was prepared from adult rats (approx. 7 weeks) and highly purified synaptic vesicles were prepared by controlled-pore glass chromatography. 12 µg of each fraction were analyzed by immunoblotting for the four SynCAM proteins as indicated on the right. The synaptic vesicle protein synaptophysin served as fractionation control. SynCAM proteins present in the preparation of crude synaptic vesicles (lane 3) were subfractionated by controlled-pore glass chromatography mainly into the CPG-void fraction (lane 5), which contains large vesicular structures and is devoid of synaptic vesicles. Consequently, SynCAM proteins were de-riched in the fraction of highly purified synaptic vesicles (lane 6). Quantitated immunoblotting demonstrated that controlled-pore glass chromatography de-riched SynCAM 1 and 4 at least 16-fold from the crude synaptic vesicle preparation and correspondingly enriches them in the void volume peak (data not shown). SynCAM 3 is de-riched from synaptic vesicles...
only approx. 6-fold by controlled-pore glass chromatography, and is the sole family members significantly present in this highly purified synaptic vesicle fraction, consistent with its report as component of synaptic vesicles (Takamori et al., 2006). Numbers on the left indicate positions of molecular weight markers. Black and white arrowheads show the running positions of the indicated SynCAM proteins in their adult and postnatal glycosylation forms, respectively. Immunoblots were a kind gift of Drs. Reinhard Jahn and Michaela Hellwig (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany).

Supplemental Figure 3. Endogenous SynCAM proteins are present at both excitatory and inhibitory synaptic specializations in dissociated hippocampal neurons.

a, Mature dissociated hippocampal neurons were fixed at 21 d.i.v. and analyzed by confocal microscopy after triple immunostaining with a pleioSynCAM antibody against SynCAMs 1, 2 and 3 (see Supplemental Figure 3), for both excitatory presynaptic markers vGlut1 and vGlut2, and for the postsynaptic inhibitory marker gephyrin. Individual immunostainings are shown in the gray scale panels as indicated. The two panels at the bottom depict the indicated double merged images (pleioSynCAM 1/2/3, red; vGlut1+2, green; gephyrin, blue). Insets in the top right of each panel represent 10-fold enlarged areas of the image shown.

b, Confocal images shown in a were analyzed using Matlab for the occurrence of single puncta and the extent of their double co-localization (SynCAM 1/2/3, 21927 puncta analyzed; vGlut1+2, 10357; gephyrin, 6622; six images). Panels depict immunostainings of the representative image shown in a after Matlab processing to define puncta. Double co-localization identifies 75 ± 6% of vGlut1+2 puncta as immunopositive for endogenous SynCAM 1/2/3, and 57 ± 4% of gephyrin puncta as immunopositive for SynCAM 1/2/3. Areas of double co-localization are depicted in the two bottom panels. Errors are stated as standard deviations. The immunostaining shown in a is representative of the quantification results.
Supplemental Figure 4. Exogenously expressed SynCAMs 1 and 2 are sorted to synapses in dissociated hippocampal neurons.

a, SynCAM 1 is localized to synapses in cultured hippocampal neurons. Extracellularly epitope-tagged SynCAM 1-flag was transfected into dissociated hippocampal neurons at 7 days in vitro (d.i.v.). After transfection, mature cultures were analyzed at 21 d.i.v. by confocal microscopy by triple immunostaining for flag, the presynaptic vesicle marker synapsin, and the postsynaptic excitatory marker PSD-95. Individual stainings are shown in the gray scale panels as indicated, and the panel on the bottom depicts the merged image (SynCAM 1-flag, red; synapsin, blue; PSD-95, green). Areas of triple co-localization appear in white. Insets in the top right of each panel represent 10-fold enlarged areas of the image shown. These confocal images were analyzed using Matlab for the number of single puncta and the occurrence of their co-localization (SynCAM 1-flag, 1918 puncta analyzed; five images). Triple co-localization identifies 45 ± 19% of SynCAM 1-flag puncta as synaptic as defined by their co-localization with both synapsin and PSD-95. Of the remaining SynCAM 1-flag puncta, approximately 1/5 are co-localized with synapsin or PSD-95 alone. Few of the SynCAM 1-flag puncta are immunonegative for either of the synaptic markers analyzed here. Errors are stated as standard deviations. Scale bar, 5 µm.

b, SynCAM 2 is localized to synapses. Extracellularly epitope-tagged SynCAM 2-flag was expressed in neurons and analyzed as described in b (SynCAM 2-flag, 933 puncta analyzed; two images). Triple co-localization identifies 67 ± 20% of SynCAM 2-flag puncta as synaptic as defined by their co-localization with both synapsin and PSD-95. Of the remaining SynCAM 2-flag puncta, approximately 1/4 are co-localized with PSD-95 alone and less with synapsin alone. Few of the SynCAM 2-flag puncta are immunonegative for either of the synaptic markers analyzed here. Scale bar, 5 µm.
Supplemental Figure 5. Endogenous SynCAM proteins are localized to synapses in dissociated hippocampal neurons.

a, Mature dissociated hippocampal neurons were fixed at 21 d.i.v. and analyzed by confocal microscopy after triple immunostaining with a pleioSynCAM antibody that detects SynCAMs 1, 2 and 3 to a comparable extent (antibody T2412, see Supplemental Fig. 1), for the presynaptic vesicle marker SV2, and for the postsynaptic excitatory marker PSD-95. Individual immunostainings are shown in the gray scale panels as indicated, and the panel at the bottom depicts the triple merged image (pleioSynCAM 1/2/3, red; SV2, green; PSD-95, blue). Areas of triple co-localization appear in white in the merged image shown in the bottom panel. Insets in the top right of each panel represent 10-fold enlarged areas of the image shown.

b, Confocal images shown in a were analyzed using Matlab for the occurrence of single puncta and the extent of their triple co-localization (SynCAM 1/2/3, 3467 puncta analyzed; SV2, 4090; PSD-95, 4090; five images). Panels depict immunostainings of the representative image shown in a after Matlab processing to define puncta. Triple co-localization identifies 70 ± 19% of endogenous SynCAM 1/2/3 puncta as immunopositive for synaptic sites as defined by their co-localization with both SV2 and PSD-95. Of the remaining staining for endogenous SynCAM 1/2/3, less than 1/10 of its puncta are co-localized with SV2 alone, and almost none with PSD-95 alone. The remaining SynCAM 1/2/3 immunopositive puncta are removed from the synaptic markers analyzed here. Areas of triple co-localization are depicted in the bottom panel.

Supplemental Figure 6. Postsynaptic overexpression of SynCAM 2 does not significantly alter mEPSC amplitudes.

mEPSC events were recorded from SynCAM 1 and 2 overexpressing hippocampal neurons and from untransduced control neurons as described in Figure 10c,d. Expression was performed using the Semliki
forest virus system for transduction of neurons. Amplitudes are not significantly altered as analyzed by Kolmogorov-Smirnov (control vs. SynCAM 1, \( D = 0.1328, p = 0.157 \); control vs. SynCAM 2, \( D = 0.0914, p = 0.324 \)).
SUPPLEMENTAL REFERENCES


Supplemental Figure 1. Characterization of SynCAM antibodies.
56x113mm (600 x 600 DPI)
Supplemental Figure 2. Subsynaptic fractionation shows that SynCAM 3, but not other SynCAM proteins, are present in synaptic vesicles.

110x177mm (600 x 600 DPI)
Supplemental Figure 3. Endogenous SynCAM proteins are present at both excitatory and inhibitory synaptic specializations in dissociated hippocampal neurons.

110x187mm (300 x 300 DPI)
Supplemental Figure 4. Exogenously expressed SynCAMs 1 and 2 are sorted to synapses in dissociated hippocampal neurons.

110x151mm (300 x 300 DPI)
Supplemental Figure 5. Endogenous SynCAM proteins are localized to synapses in dissociated hippocampal neurons.

110x157mm (300 x 300 DPI)
Supplemental Figure 6. Postsynaptic overexpression of SynCAM 2 does not significantly alter mEPSC amplitudes.