SynCAM 1 participates in axo-dendritic contact assembly and shapes neuronal growth cones

Massimiliano Stagi, Adam I. Fogel, and Thomas Biederer[1]

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520

Edited* by Pietro De Camilli, Yale University and Howard Hughes Medical Institute, New Haven, CT, and approved March 9, 2010 (received for review October 13, 2009)

Neuronal growth cones are highly motile structures that tip developing neurites and explore their surroundings before axo-dendritic contact and synaptogenesis. However, the membrane proteins organizing these processes remain insufficiently understood. Here we identify that the synaptic cell adhesion molecule 1 (SynCAM 1), an immunoglobulin superfamily member, is already expressed in developing neurons and localizes to their growth cones. Upon interaction of growth cones with target neurites, SynCAM 1 rapidly assembles at these contacts to form stable adhesive clusters. Synaptic markers can also be detected at these sites. Addressing the functions of SynCAM 1 in growth cones preceding contact, we determine that it is required and sufficient to restrict the number of active filopodia. Further, SynCAM 1 negatively regulates the morphological complexity of migrating growth cones. Focal adhesion kinase, a binding partner of SynCAM 1, is implicated in its morphogenetic activities. These results reveal that SynCAM 1 acts in developing neurons to shape migrating growth cones and contributes to the adhesive differentiation of their axo-dendritic contacts.

CADM | focal adhesion kinase | growth cone | synaptic adhesion | synaptogenesis

Growth cones tip differentiating neurites and target exploration occurs through filopodia (1–3). Upon contact, axonal growth cones undergo a rapid morphological transition that initiates synaptic membrane differentiation in conjunction with the appearance of synaptic vesicles, electron-dense cleft material, and postsynaptic specializations (4–6). Although the cytoskeletal framework of growth cones is being defined (7, 8), the best understood roles of surface proteins are in outgrowth and guidance (9, 10). The roles of membrane proteins in shaping growth cones and target exploration remain less well defined.

In contrast, insight has been gained into the roles of surface proteins in synaptic differentiation. Trans-synaptic interactions of synaptic cell adhesion molecules (SynCAMs), neurexins/neuroligins, ephrinB/EphB receptors, and select other proteins organize developing synapses (11, 12). Additional proteins act in synapse maturation, notably N-cadherin (13). Although conceptually intriguing, no evidence points to roles of these proteins in axo-dendritic contact differentiation.

SynCAM 1, alternatively named CADM1/IGSF4/nectin-like 2 (14, 15), is an Ig adhesion molecule that drives synapse formation in developing neurons. SynCAM 1 is already expressed in the late embryonic and early postnatal brain, whereas the other SynCAM family members as well as neurexins and neuroligins peak subsequently during synaptogenesis (16–19). This profile of SynCAM 1 indicates functions preceding synapse formation. We now reveal SynCAM 1 as a surface protein of axonal growth cones that assembles rapidly and stably at axo-dendritic contacts. Sites marked by SynCAM 1 can also contain synaptic markers, indicating that they have the potential to differentiate into nascent synapses. Before contact, SynCAM 1 regulates the complexity of growth cones and controls their active filopodia number, and we identify focal adhesion kinase (FAK) as a binding partner and effector in shaping growth cones. These results demonstrate that SynCAM 1 is an early player in axo-dendritic contact differentiation and organizes growth cones through a FAK-dependent pathway.

Results

Growth Cones Express SynCAM 1. To elucidate the early developmental roles of SynCAM 1, we analyzed its expression in dissociated hippocampal neurons at 5 days in vitro (d.i.v.). At this time, axons are specified and dendrites have begun to grow, but most synapses have yet to form (20). SynCAM 1 is already prominently expressed at this stage, preceding other synaptic adhesion molecules (Fig. 1*A*), and is enriched in growth cones of neurites positive for the axonal marker tau (Fig. 1*B* and Fig. S1*B*). SynCAM 1 knockout controls confirm antibody specificity (21) (Fig. 1*C* and Fig. S1 *A* and *C*). These results agree with the presence of SynCAM 1 in growth cone preparations (Fig. S2) and with the recent proteomic identification of SynCAM 1 as a strongly enriched growth cone protein (22).

We next visualized SynCAM 1 in live growth cones by inserting the pH-sensitive GFP variant pHluorin (23) into the extracellular domain (see Fig. 3*B* for a model). This construct is functional as it rescues SynCAM 1 knockout phenotypes in immature neurons and is properly localized to mature synapses (see below). Live imaging of migrating growth cones identifies SynCAM 1–pHluorin in their central region and filopodia (Fig. S3), similar to endogenous SynCAM 1. To analyze the surface expression of SynCAM 1–pHluorin, we imaged growth cones while transiently lowering the extracellular pH to quench its surface-exposed pool. This leaves intracellular pHluorin molecules unaffected (Fig. S4 *A* and *B*). SynCAM 1–pHluorin fluorescence in growth cones and their filopodia are almost lost at low extracellular pH, demonstrating that SynCAM 1 is expressed on the growth cone surface (Fig. S4 *C*–*E*). We next addressed what fraction of SynCAM 1 is surface-exposed by imaging growth cones first live at neutral and then at low pH, followed by fixation, permeabilization and repeat imaging at neutral pH (Fig. 1*D*). SynCAM 1–pHluorin fluorescence was indistinguishable at both neutral pH conditions (Fig. 1*E*), demonstrating that only a small fraction resides intracellularly.

Rapid Assembly of SynCAM 1 upon Axo-Dendritic Contact. Filopodia participate in the rapid formation of presynaptic specializations as axonal growth cones pass dendrites (1, 24, 25). Considering the roles of SynCAM 1 in cell adhesion, we asked whether it participates in these axo-dendritic interactions of growth cones. By selecting growth cones expressing SynCAM 1–pHluorin in approach to SynCAM 1–pHluorin–marked neurites, we find that axo-

Author contributions: M.S., A.I.F., and T.B. designed research; M.S., A.I.F., and T.B. performed research; M.S., A.I.F., and T.B. analyzed data; and T.B. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]To whom correspondence should be addressed. E-mail: thomas.biederer@yale.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0911798107/DCSupplemental.

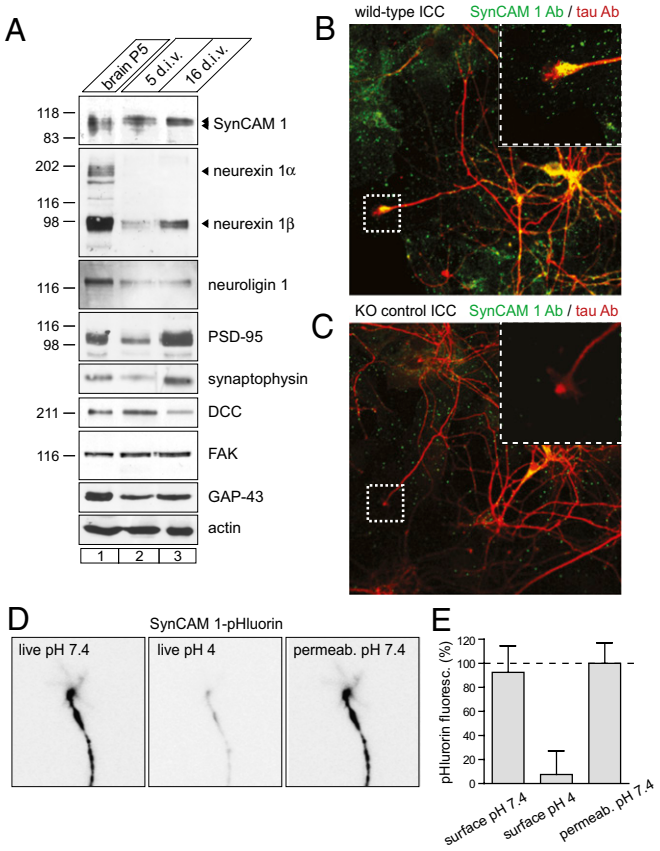

Fig. 1. SynCAM 1 localizes to growth cones. (*A*) Immunoblot analysis of neuronal culture lysates at the indicated days in vitro (d.i.v.). SynCAM 1 expression precedes the synaptic adhesion molecules neurexin and neuroligin. PSD-95 and synaptophysin are synaptic protein controls, whereas DCC, FAK, and GAP-43 are already expressed in growth cones. Actin served as loading control, and rat forebrain from postnatal day 5 (P5) as positive control. (*B* and *C*) Confocal fluorescence image of dissociated mouse wild-type (*B*) and SynCAM 1 knockout (KO; *C*) hippocampal neurons at 5 d.i.v. after immunostaining for SynCAM 1 (green) and tau (red). Specific SynCAM 1 staining is detected in growth cones. Boxes marks representative growth cones enlarged in the *Inset*. (*D*) SynCAM 1 is predominantly present on growth cone surfaces. Dissociated rat hippocampal neurons expressing extracellularly tagged SynCAM 1–pHluorin were imaged live at 5 d.i.v. at pH 7.4 (*Left*) and then transiently at pH 4 (*Center*) to quench the pHluorin surface signal. To detect the total pool of SynCAM 1–pHluorin, the same growth cone was permeabilized with 0.1% Triton X-100 containing fixative, washed, and imaged again at neutral pH using the same settings (*Right*). (*E*) Quantification of SynCAM 1–pHluorin fluorescence intensity obtained as in *D* ($n = 3$).

dendritic contact triggers the threefold accumulation of SynCAM 1 at contacts within 5 min (Fig. 2 *A*–*C* and Movie S1). No volumetric membrane increases occur at these sites (Fig. S5). Interestingly, SynCAM 1 assembly not only is initiated quickly, but also is completed rapidly, as its amount increases only marginally subsequent to contact (Fig. 2*B*, $t_{45\ min}$). These SynCAM 1 assemblies persist once the growth cone migrates onward (Fig. 2*A*, $t_{90\ min}$).

We next analyzed contacts between growth cones and neurites that both express SynCAM 1ΔIg1–pHluorin, a construct lacking the first Ig domain required for adhesion (14). This construct replicates the position of pHluorin in SynCAM 1–pHluorin and is expressed on growth cone surfaces (Fig. S6). Notably, this adhesion-deficient SynCAM 1 does not accumulate upon contact (Fig. 2 *D* and *E*). Adhesive interactions therefore underlie the contact assembly of SynCAM 1.

To better define this assembly of SynCAM 1, we separately transfected neurons with SynCAM 1–pHluorin to detect it in growth cones or with soluble Cherry to label neurites and cultured them together. Live imaging shows that SynCAM 1–pHluorin strongly accumulates in growth cones upon contact with Cherry-positive neurites (Fig. 2 *F* and *G*). These results demonstrate that SynCAM 1 is a growth cone adhesion protein that assembles rapidly and stably at axo-dendritic contacts.

Differentiation of SynCAM-Marked Axo-Dendritic Contact Sites. We next asked whether SynCAM 1 assemblies mark differentiating growth cone contacts. Neurons were transfected with SynCAM 1–Cherry to detect it in growth cones or with GFP-tagged PSD-95 to label dendritic clusters, plated together, and analyzed (Fig. 2*H* and Fig. S7). Endogenous PSD-95 is already expressed at low levels in these immature neurons (see also Fig. 1*A*) and can be colocalized with the comparatively low number of developing synaptic contacts (26). Live imaging shows that growth cone filopodia containing SynCAM 1 are apposed to dendritic PSD-95 clusters. We frequently observed that these growth cone assemblies of SynCAM 1 remain juxtaposed to PSD-95-positive sites for hours (Fig. 2*H*), and we recorded the gradual accumulation of PSD-95 at these contacts (Fig. S7). These stable sites also contain other presynaptic proteins as shown by post-hoc immunostaining for synaptotagmin I (Fig. 2*I*). In consequence, discrete SynCAM 1 assemblies are retained along axonal crossing points with dendrites, and these sites remain stably apposed to PSD-95 clusters (Fig. 2*J*), with a subset containing presynaptic markers (Fig. 2*J*). Consistent with a continued differentiation of these sites, SynCAM 1 is localized to synapses in mature neurons (Fig. S8).

SynCAM 1 Restricts the Structural Organization of Migrating Growth Cones. Does SynCAM 1 function in growth cones before axo-dendritic contact? In support of morphogenetic roles, we observed that live growth cones containing elevated SynCAM 1 appear less dynamic than controls expressing myristoylated GFP (myrGFP) as a membrane marker (Fig. 3 *A* and *B* and Movie S2). These optical recordings were acquired under nonlinear, high-gain conditions to trace the complete plasma membrane, unlike the analysis of SynCAM 1 localization under normal gain in Fig. 2. We first determined the number of growth cone filopodia that alter their length or position throughout the optical recording, scoring those as "active," and show that elevated SynCAM 1 strongly reduces their number to 48 ± 11% of control levels (Fig. 3*E*). We next determined the complexity of growth cones by Sholl analysis, an algorithm to assess the general complexity of structures (27). Because myrGFP and SynCAM 1–pHluorin delineate identical growth cone outlines (Fig. S9 *A* and *B*), their comparative analysis was performed. This demonstrated that exogenous SynCAM 1–pHluorin lowers growth cone complexity to 52 ± 7% of control levels (Fig. 3*F*), consistent with their simpler appearance. In agreement, exogenous SynCAM 1 reduces growth cone perimeters to 49 ± 16% of control levels. SynCAM 1 is therefore sufficient to restrict growth cone filopodial dynamics and membrane complexity.

SynCAM 1 Shapes Growth Cones Through FERM Interactions. These morphogenetic roles of SynCAM 1 in growth cones pointed to interactions with cytoskeletal regulators. Such interactions can be mediated by an intracellular motif of SynCAMs predicted to bind FERM (protein 4.1/ezrin/radixin/moesin) domains (28) (Fig. 3*D*), which are present in a number of cytoskeletal components such as the SynCAM-binding partner protein 4.1B (29–31). To test this possibility, we generated a SynCAM 1ΔFERM–pHluorin mutant lacking five amino acids in this motif (Fig. 3 *C* and *D*). This deletion prevents FERM domain interactions (see Fig. 4*A*) without altering SynCAM 1 expression in growth cone membranes as assessed from tracing studies (Fig. S9 *C* and *D*). Interestingly, this ΔFERM mutation abrogates the effects of elevated SynCAM 1 on active filopodia number (Fig. 3*E*) and on

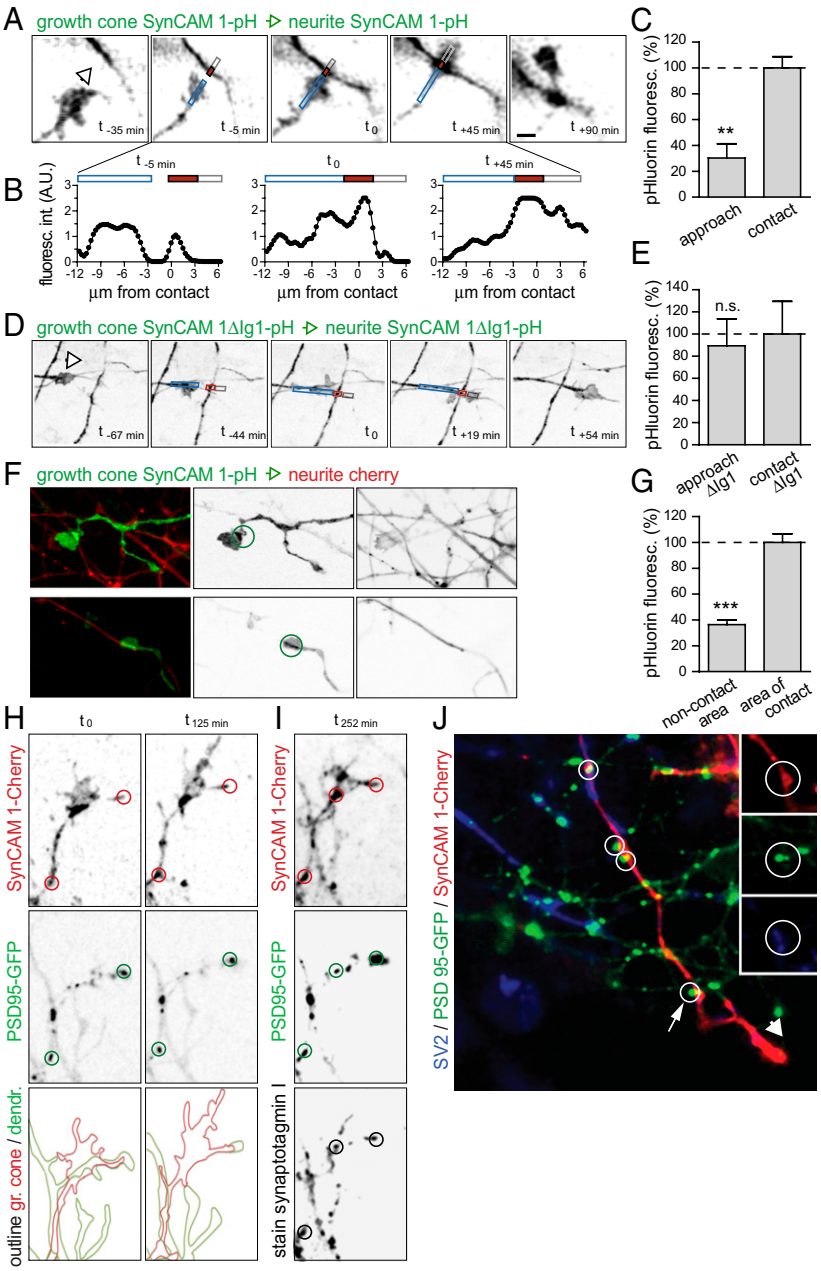

Fig. 2. Rapid and persistent assembly of SynCAM 1 during axo-dendritic contact. (*A*) Rat hippocampal growth cones expressing SynCAM 1–pHluorin were imaged at 5 d.i.v. as they advanced toward a dendrite. The arrowhead marks the growth cone direction. Frames were obtained every 5 min. Bars indicate the areas quantitated in *B*. (Scale bar in *A* and *D*: 10 µm.). See Movie S1. (*B*) Line scan analysis of SynCAM 1–pHluorin distribution. Colored bars on top correspond to the quantitated areas in *A*. (*C*) Axo-dendritic contact increases SynCAM 1–pHluorin threefold. Fluorescence intensities were determined as in *B* before and during contact ($P = 0.008$; $n = 3$). (*D* and *E*) SynCAM 1 accumulation at contacts requires adhesion. Neurons expressing adhesion-deficient SynCAM 1ΔIg1–pHluorin were imaged as described in *A* and *C* ($n = 4$). n.s., not significant. (*F*) SynCAM 1–pHluorin accumulates in growth cones. Dissociated rat hippocampal neurons were transfected with SynCAM 1–pHluorin (green) or soluble Cherry (red), cultured together, and imaged live at 5 d.i.v. The two representative examples show the accumulation of SynCAM 1–pHluorin at growth cone contacts with Cherry-marked neurites. (*G*) SynCAM 1–pHluorin fluorescence signals were collected as in *F* from growth cone areas in contact with a neurite and from the proximal noncontact areas. SynCAM 1–pHluorin signal is increased 2.5-fold at the contact ($P < 0.0001$; $n = 4$). (*H*) Growth cone SynCAM 1 marks differentiating axo-dendritic contacts. Rat hippocampal neurons were transfected with SynCAM 1–Cherry (red) or PSD95-GFP (green), cultured together, and imaged live at 5 d.i.v. Panels depict the same field of view. Signals were traced to outline the growth cone and dendrites (*Bottom* row). *Left* panels depict a SynCAM 1-expressing growth cone that has crossed a dendrite (t_0). Circles mark stable contacts with dendritic PSD-95 clusters (*Right* panels, $t_{125\ min}$). (*I*) The culture imaged in *H* was fixed at $t_{252\ min}$. Immunostaining for synaptotagmin I shows this presynaptic marker at SynCAM 1/PSD-95 positive contacts. (*J*) Neurons were separately transfected and analyzed as in *H*. The arrowhead indicates the growth cone direction. Circles mark apposed axonal SynCAM 1 clusters with PSD-95 at dendritic crossing points that were stable over 6 h. Post-hoc immunostaining identifies SV2 (blue) at a subset of these clusters (marked by circles). *Insets* show individual channels of the circled area marked by an arrow.

growth cone complexity (Fig. 3*F* and Movie S2). FERM domain interactions of SynCAM 1 are therefore critical to its organization of growth cones.

Endogenous SynCAM 1 Is Required to Reduce Growth Cone Dynamics and Complexity. Does endogenous SynCAM 1 organize growth cone structure? We addressed this by imaging growth cones of SynCAM 1 knockout and wild-type neurons that expressed actin tagged with the fluorescent Cherry protein to visualize their cytoskeleton. Intriguingly, growth cones lacking SynCAM 1 are more exuberant and have more than double the number of active filopodia than wild-type controls (Fig. 3 *G–I*). Further, growth cones lacking SynCAM 1 are more complex (Fig. 3*J*). Re-expression of SynCAM 1–pHluorin rescues these phenotypes (Fig. 3 *I* and *J*). The effects of exogenous SynCAM 1 in these rescued mouse knockout neurons are less pronounced than in rat neurons. This may reflect a dependency on the total SynCAM 1 dose that is higher when overexpressed in wild-type cells or a stronger exogenous expression in rat neurons. Together, these results identify endogenous SynCAM 1 as a negative regulator of the structural complexity and filopodial dynamics of growth cones.

FAK Is a Partner of SynCAM 1. We hypothesized that the non-receptor tyrosine kinase FAK mediates the morphogenetic effects of SynCAM 1 as it contains a FERM domain, is expressed in hippocampal growth cones (32), and negatively regulates membrane protrusions in nonneuronal cells (33). Further, FAK affects migrating growth cones through controlling their substrate contacts (34). FAK is best characterized in fibroblasts, where it is recruited to integrin adhesion sites to regulate the turnover of focal adhesions, large complexes that connect the extracellular matrix to the cytoskeleton (35, 36).

To test FAK interactions, we performed affinity chromatography of forebrain extracts on the SynCAM 1 cytosolic sequence. This identifies the strong retention of FAK (Fig. 4*A*, lane 2). To

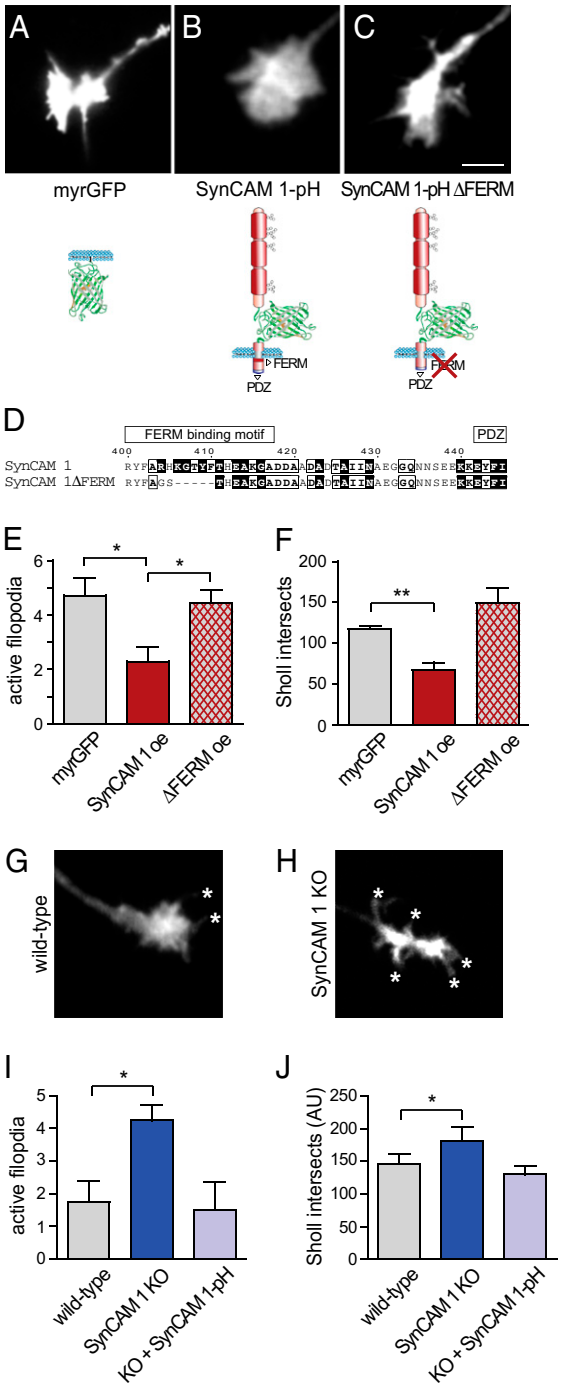

Fig. 3. SynCAM 1 reduces active filopodia number and growth cone complexity. (*A*–*C*) Exogenous SynCAM 1 reduces growth cone complexity in dependence on its FERM-binding motif. Rat hippocampal neurons expressing myristoylated GFP (myrGFP) as membrane marker (*A*), SynCAM 1–pHluorin (*B*), or a mutant in the FERM motif (SynCAM 1–pH ΔFERM; *C*) were imaged live at 5 d.i.v. Images were acquired under nonlinear conditions to detect the total fluorescence signal. Models depict the fusion proteins. The illustration of GFP was used with permission from Roger Tsien, University of California, San Diego. See Movie S2. (Scale bar in *A*–*C*: 3 μm.) (*D*) Alignment of the cytosolic sequence of SynCAM 1 (*Upper*) and the ΔFERM mutation (*Lower*). (*E*) Active filopodia number of growth cones imaged as in *A*–*C* is decreased to 48 ± 11% by exogenous SynCAM 1–pHluorin in dependence on FERM interactions (myrGFP vs. SynCAM 1–pH, $P = 0.013$; SynCAM 1–pH vs. SynCAM 1–pH ΔFERM; $P = 0.011$; $n = 7$). oe, overexpression. (*F*) Sholl analysis of growth cones imaged as in *A*–*C* demonstrates that exogenous SynCAM 1 decreases complexity to 52 ± 7% ($P = 0.008$). SynCAM 1 lacking the FERM motif has no activity ($n = 5$). (*G* and *H*) Lack of SynCAM 1 increases apparent growth cone complexity. Wild-type (*G*) or SynCAM 1 knockout (*H*) mouse hippocampal growth cones were imaged live at 5 d.i.v. using actin–Cherry to label membrane protrusions. Asterisks mark filopodia. (*I*) Growth cones lacking SynCAM 1 contain 2.4-fold more active filopodia (wild type vs. KO, $P = 0.019$; $n = 4$). This phenotype is rescued by exogenous SynCAM 1-pHluorin. Growth cones were imaged as in *G* and *H*, obtaining frames every 5 s for 50 s. (*J*) Sholl analysis of growth cones imaged as in *G* and *H* demonstrates that the lack of SynCAM 1 increases complexity by 20% ($P = 0.027$; $n = 3$). SynCAM 1–pHluorin rescues ($n = 4$).

map this interaction, we employed a ΔPDZ mutant of SynCAM 1 lacking the three carboxyl-terminal amino acids, as well as the ΔFERM deletion described above. As expected, FAK binds SynCAM 1 independently of PDZ domains (lane 3), but requires the FERM motif (lane 4). This interaction is specific, as the FERM domain containing protein talin is not retained. The PDZ domain protein CASK served as positive control (14). FAK binds more strongly to SynCAM 1 than to neurexin I, which also contains a motif predicted to bind FERM domains, which supports a select role of the SynCAM 1/FAK interaction (Fig. S10). Correspondingly, SynCAM 1 extracted from forebrain bound to the FERM domain of FAK (Fig. 4*B*). The coimmunoprecipitation of flag-tagged SynCAM 1 with FAK from COS7 cells supports their direct binding (Fig. 4*C*). Coimmunoprecipitation from brain could not be performed due to the low precipitation yield of the anti-SynCAM 1 antibodies.

Colocalization analyses corroborate these biochemical studies. Total internal reflection fluorescence (TIRF) imaging of live COS7 cells coexpressing GFP–FAK and SynCAM 1–Cherry demonstrates that both proteins colocalize at the plasma membrane (Fig. 4*D*). Interestingly, TIRF microscopy of growth cones shows that both proteins are present together in the central region and filopodia (Fig. 4*E*), where they dynamically colocalize (Movie S3). Here, motile packets containing SynCAM 1 and FAK sort rapidly from the central region into filopodia at 15 ± 7 μm/min ($n = 3$). These results are consistent with direct interactions of SynCAM 1 and its partner FAK at the growth cone membrane.

SynCAM 1 Signals via FAK in Growth Cones. We next addressed whether FAK also is a functional effector of SynCAM 1. These studies used a dominant-negative FAK construct that lacks the FERM and kinase domains, termed FAK-related nonkinase (FRNK), which reduces FAK signaling probably via competitive binding to its partners (37, 38). This revealed that the effects of SynCAM 1 on growth cone complexity require FAK signaling (Fig. 5*A*). Additionally, the restriction of active filopodia number by SynCAM 1 (myrGFP, 4.7 ± 1.7 active filopodia; SynCAM 1–pH, 2.3 ± 1.4 active filopodia; $P = 0.013$; $n = 7$) was blocked by FRNK (SynCAM 1–pH + FRNK, 3.7 ± 0.7 active filopodia; $n = 7$). FAK-independent pathways likely act in concert as FRNK alone is not sufficient to reduce the number of active filopodia (FRNK, 5.1 ± 0.6 active filopodia; $n = 5$) and complexity (Fig. 5*A*). Exogenous SynCAM 1 therefore requires FAK to shape growth cones, presumably by overriding endogenous pathways.

Finally, we addressed whether SynCAM 1 alters FAK activity in growth cones prepared from wild-type and SynCAM 1 knockout forebrains at postnatal day 5. Interestingly, loss of SynCAM 1 reduces the specific activity of FAK in growth cones by 22 ± 6% as determined after quantitative immunoblotting with antibodies against autophosphorylated, active FAK and total FAK (Fig. 5*B*). Together, FAK is required for the morphogenetic activities of SynCAM 1, and SynCAM 1 alters FAK activity in growth cones.

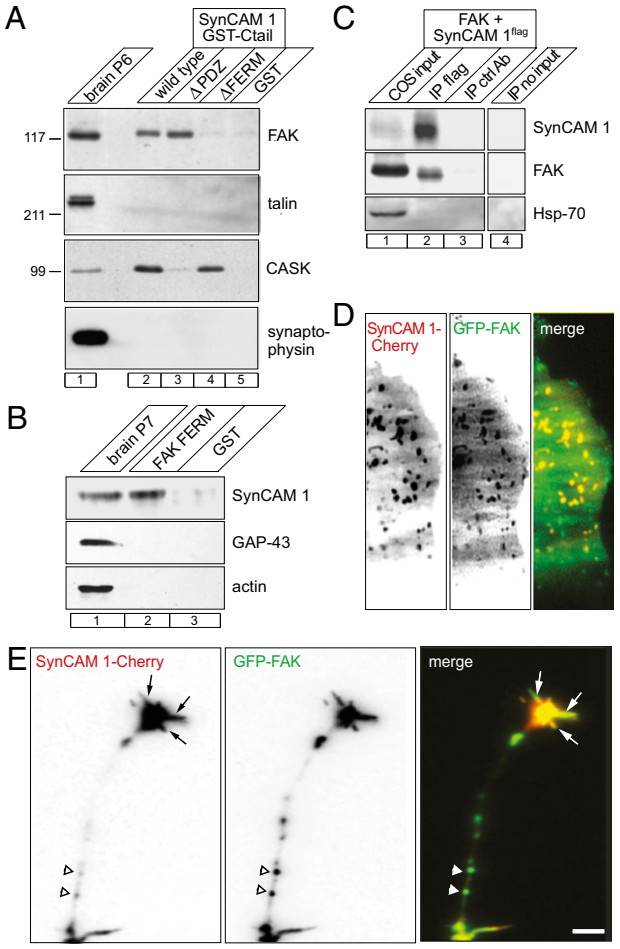

Fig. 4. FAK is a binding partner of SynCAM 1. (*A*) FAK binds the intracellular FERM motif of SynCAM 1. Rat forebrain proteins were solubilized at P5 and incubated with equal amounts of GST fusions of the cytosolic sequence of SynCAM 1, a ΔPDZ construct, or its ΔFERM mutant. GST served as control for nonspecific binding (lane 5). Eluate lanes contain $^{1}/_{40}$ of the input shown. (*B*) Binding of SynCAM 1 to the FERM domain of FAK. Rat forebrain proteins extracted at P7 were bound to a GST fusion of the FERM domain of FAK, demonstrating SynCAM 1 retention. GAP-43 and actin served as negative controls, and GST as control for nonspecific binding. Eluate lanes contain $^{1}/_{50}$ of the input shown. (*C*) Direct interaction of SynCAM 1 and FAK. Flag-tagged SynCAM 1 and FAK coexpressed in COS7 cells coimmunoprecipitate with anti-flag antibodies. Immunoprecipitates contain $^{1}/_{25}$ of the input shown. (*D*) SynCAM 1 and FAK colocalize in the plasma membrane as imaged by TIRF microscopy of live COS7 cells coexpressing SynCAM 1–Cherry (red) and GFP-FAK (green). (*E*) TIRF microscopy of live rat hippocampal growth cones coexpressing GFP–FAK (green) and SynCAM 1-Cherry (red). The merged image shows their colocalization in the central growth cone region and its filopodia (arrows) and in apparent axonal transport packets (arrowheads). See Movie S3. (Scale bar: 10 μm.)

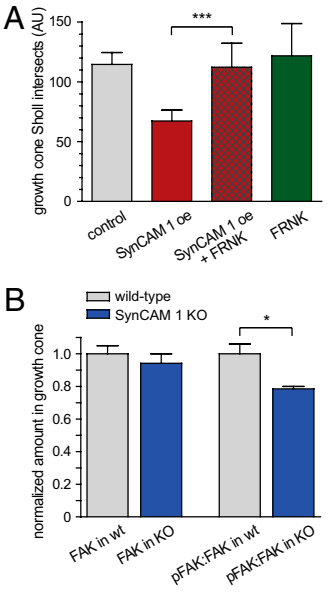

Fig. 5. FAK is a signaling partner of SynCAM 1. (*A*) Dominant-negative inhibition of FAK by FRNK blocks exogenous SynCAM 1–pHluorin from restricting complexity as shown by Sholl analysis (SynCAM 1–pH vs. SynCAM 1–pH plus FRNK, $P = 0.001$; $n = 4$). oe, overexpression. (*B*) Loss of SynCAM 1 reduces specific FAK activity in growth cones by 22 ± 6% ($P = 0.025$; $n = 3$) while not affecting FAK enrichment in growth cones. Growth cones were prepared from wild-type and SynCAM 1 knockout mice at P7. Equal protein amounts were analyzed by quantitative immunoblotting for autophosphorylated and total FAK. Loaded amounts were normalized to GAP-43.

Discussion

This study demonstrates that SynCAM 1 performs successive functions in developing neurons from shaping growth cones to the assembly of axo-dendritic contacts. These properties are distinct from other proteins like N-cadherin, which is mostly absent from growth cones and accumulates at synapses only after the growth cone has migrated on (39, 40), and are not shared by L1 and NCAM. These Ig proteins act in axon outgrowth and guidance (9), and SynCAM 1 additionally contributes to these growth-cone–dependent processes as axonal pathfinding errors occur when its expression is reduced in chicken (41). Although neurexins have also been detected in growth cones (42), they have not been characterized in this compartment.

A key finding of this study is that SynCAM 1 assembles at growth cone contacts with target neurites. This likely involves its lateral clustering, but localized exocytosis may also contribute to its delivery to axo-dendritic contacts. We presume that SynCAM 1 clusters primarily engage in homophilic adhesion, as its heterophilic partner SynCAM 2 is less prominently expressed in the early postnatal hippocampus (16). SynCAM 2 expression increases in development, and its binding to SynCAM 1 could later refine these nascent sites. During these stages of synaptic differentiation, SynCAM adhesion may act in concert with other trans-synaptic adhesion molecules, such as neurexins/neuroligins (42–45).

Our study provides additional insight into the organization of migrating growth cones and finds that SynCAM 1 reduces the number of active filopodia. This is relevant as the regulation of filopodia by membrane proteins is insufficiently understood compared to the cytoskeletal machinery controlling these protrusions (46, 47). Interestingly, the restriction of active growth cone filopodia number by SynCAM 1 is converse to the postsynaptic effects of EphB receptors, which are required for proper dendritic filopodia motility (48). The expression levels of these membrane proteins may therefore mutually regulate the extent of axo-dendritic target exploration. Overall, this reduction in the membrane complexity of growth cones by elevated SynCAM 1 could result in their increased ability to maintain target contacts and differentiate them into synapses.

With respect to intracellular interactions, our results show that FAK is a binding partner and effector of SynCAM 1 in the shaping of migrating growth cones. This makes SynCAM 1 one of the few membrane proteins that directly bind FAK, together with EGF and PDGF receptors and possibly integrins (49, 50), NCAM 140 (51), and EphA receptors (52). FAK can be activated by engagement of its FERM domain (53), and SynCAM 1 binding may localize and spatially define FAK activity within growth cones. This would be consistent with the reduced specific FAK activity in growth cones lacking SynCAM 1. Interestingly, FAK restricts the

number of complex synapses in mature neurons (54), and future studies will determine whether a SynCAM–FAK complex operates at synapses subsequent to axo-dendritic contact.

Materials and Methods

An extended section is provided in *SI Materials and Methods* and Table S1.

Biochemical Studies. Rat forebrain homogenate was fractionated at P5–P7 (55). Affinity chromatography was performed as described (14).

Neuronal Cell Culture. Dissociated hippocampal neurons were cultured at postnatal day P0 or P1 (56). Mouse neuronal cultures were prepared from SynCAM 1 knockout mice (21) and compared to wild-type littermate controls.

Live Imaging. Neuronal cultures were imaged live at 5–6 d.i.v. in modified Tyrode solution (56) on an Olympus Ix81 microscope with an autofocus system or on a Perkin-Elmer UltraView Spinning Disk microscope. TIRF imaging was performed on the Olympus Ix81 microscope. Images were obtained using a low-intensity laser line and low exposure to reduce phototoxicity.

Statistical analyses were performed using two-tailed *t* tests, and statistical errors correspond to SEM unless indicated otherwise.

ACKNOWLEDGMENTS. We thank Drs. A. Koleske, E. Stein, S. Strittmatter, and S. Chandra for discussions. We are grateful to Dr. T. Momoi (National Institute for Neuroscience, Tokyo) for generously providing SynCAM 1 knockout mice; Drs. C. Damsky (University of California at San Francisco) and D. Schlaepfer (University of California at San Diego) for FAK and FRNK vectors; Drs. B. Serrels and M. Frame (University of Edinburgh) for the GST–FAK FERM construct; Dr. D. Bredt (Lilly Research Laboratories) for the PSD-95 vector; and the Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University, for access to its imaging core. This work was supported by National Institutes of Health Grant R01 DA018928 (to T.B.), a Dana Foundation Brain and Immuno-Imaging Grant (to T.B.), and a Brown-Coxe Fellowship (to M.S.).

1. Cooper MW, Smith SJ (1992) A real-time analysis of growth cone-target cell interactions during the formation of stable contacts between hippocampal neurons in culture. *J Neurobiol* 23:814–828.
2. Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17:91–102.
3. Fiala JC, Feinberg M, Popov V, Harris KM (1998) Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J Neurosci* 18:8900–8911.
4. Rees RP, Bunge MB, Bunge RP (1976) Morphological changes in the neuritic growth cone and target neuron during synaptic junction development in culture. *J Cell Biol* 68:240–263.
5. Ahmari SE, Smith SJ (2002) Knowing a nascent synapse when you see it. *Neuron* 34: 333–336.
6. Jin Y, Garner CC (2008) Molecular mechanisms of presynaptic differentiation. *Annu Rev Cell Dev Biol* 24:237–262.
7. Lowery LA, Van Vactor D (2009) The trip of the tip: Understanding the growth cone machinery. *Nat Rev Mol Cell Biol* 10:332–343.
8. Pak CW, Flynn KC, Bamburg JR (2008) Actin-binding proteins take the reins in growth cones. *Nat Rev Neurosci* 9:136–147.
9. Maness PF, Schachner M (2007) Neural recognition molecules of the immunoglobulin superfamily: Signaling transducers of axon guidance and neuronal migration. *Nat Neurosci* 10:19–26.
10. Suter DM, Errante LD, Belotserkovsky V, Forscher P (1998) The Ig superfamily cell adhesion molecule, apCAM, mediates growth cone steering by substrate-cytoskeletal coupling. *J Cell Biol* 141:227–240.
11. Dalva MB, McClelland AC, Kayser MS (2007) Cell adhesion molecules: Signalling functions at the synapse. *Nat Rev Neurosci* 8:206–220.
12. Biederer T, Stagi M (2008) Signaling by synaptogenic molecules. *Curr Opin Neurobiol* 18:261–269.
13. Arikkath J, Reichardt LF (2008) Cadherins and catenins at synapses: Roles in synaptogenesis and synaptic plasticity. *Trends Neurosci* 31:487–494.
14. Biederer T, et al. (2002) SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525–1531.
15. Shingai T, et al. (2003) Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. *J Biol Chem* 278:35421–35427.
16. Thomas LA, Akins MR, Biederer T (2008) Expression and adhesion profiles of SynCAM molecules indicate distinct neuronal functions. *J Comp Neurol* 510:47–67.
17. Fogel AI, et al. (2007) SynCAMs organize synapses through heterophilic adhesion. *J Neurosci* 27:12516–12530.
18. Kang Y, Zhang X, Dobie F, Wu H, Craig AM (2008) Induction of GABAergic postsynaptic differentiation by alpha-neurexins. *J Biol Chem* 283:2323–2334.
19. Song JY, Ichtchenko K, Südhof TC, Brose N (1999) Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc Natl Acad Sci USA* 96:1100–1105.
20. Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. *J Neurosci* 8:1454–1468.
21. Fujita E, et al. (2006) Oligo-astheno-teratozoospermia in mice lacking RA175/TSLC1/SynCAM/IGSF4A, a cell adhesion molecule in the immunoglobulin superfamily. *Mol Cell Biol* 26:718–726.
22. Nozumi M, et al. (2009) Identification of functional marker proteins in the mammalian growth cone. *Proc Natl Acad Sci USA* 106:17211–17216.
23. Miesenböck G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192–195.
24. Meyer MP, Smith SJ (2006) Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. *J Neurosci* 26: 3604–3614.
25. Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat Neurosci* 3:445–451.
26. Anderson TR, Shah PA, Benson DL (2004) Maturation of glutamatergic and GABAergic synapse composition in hippocampal neurons. *Neuropharmacology* 47:694–705.
27. Sholl DA (1953) Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat* 87:387–406.
28. Biederer T (2006) Bioinformatic characterization of the SynCAM family of immunoglobulin-like domain-containing adhesion molecules. *Genomics* 87:139–150.
29. Hoy J, Constable J, Vicini S, Fu Z, Washbourne P (2009) SynCAM1 recruits NMDA receptors via protein 4.1B. *Mol Cell Neurosci* vol 42:466–483.
30. Diakowski W, Grzybek M, Sikorski AF (2006) Protein 4.1, a component of the erythrocyte membrane skeleton and its related homologue proteins forming the protein 4.1/FERM superfamily. *Folia Histochem Cytobiol* 44:231–248.
31. Chishti AH, et al. (1998) The FERM domain: A unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem Sci* 23:281–282.
32. Burgaya F, Menegon A, Menegoz M, Valtorta F, Girault JA (1995) Focal adhesion kinase in rat central nervous system. *Eur J Neurosci* 7:1810–1821.
33. Chan KT, Cortesio CL, Huttenlocher A (2009) FAK alters invadopodia and focal adhesion composition and dynamics to regulate breast cancer invasion. *J Cell Biol* 185: 357–370.
34. Robles E, Gomez TM (2006) Focal adhesion kinase signaling at sites of integrin-mediated adhesion controls axon pathfinding. *Nat Neurosci* 9:1274–1283.
35. Mitra SK, Hanson DA, Schlaepfer DD (2005) Focal adhesion kinase: In command and control of cell motility. *Nat Rev Mol Cell Biol* 6:56–68.
36. Parsons JT, Martin KH, Slack JK, Taylor JM, Weed SA (2000) Focal adhesion kinase: A regulator of focal adhesion dynamics and cell movement. *Oncogene* 19:5606–5613.
37. Richardson A, Parsons T (1996) A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. *Nature* 380:538–540.
38. Schaller MD, Borgman CA, Parsons JT (1993) Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Mol Cell Biol* 13:785–791.
39. Benson DL, Tanaka H (1998) N-cadherin redistribution during synaptogenesis in hippocampal neurons. *J Neurosci* 18:6892–6904.
40. Jontes JD, Emond MR, Smith SJ (2004) In vivo trafficking and targeting of N-cadherin to nascent presynaptic terminals. *J Neurosci* 24:9027–9034.
41. Niederkofler V, Baeriswyl T, Ott R, Stoeckli ET (2010) Nectin-like molecules/SynCAMs are required for post-crossing commissural axon guidance. *Development* 137: 427–435.
42. Dean C, et al. (2003) Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* 6:708–716.
43. Nam CI, Chen L (2005) Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc Natl Acad Sci USA* 102:6137–6142.
44. Gerrow K, et al. (2006) A preformed complex of postsynaptic proteins is involved in excitatory synapse development. *Neuron* 49:547–562.
45. Graf ER, Zhang X, Jin S-X, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119:1013–1026.
46. Gupton SL, Gertler FB (2007) Filopodia: The fingers that do the walking. *Sci STKE* 2007:re5.
47. Faix J, Rottner K (2006) The making of filopodia. *Curr Opin Cell Biol* 18:18–25.
48. Kayser MS, Nolt MJ, Dalva MB (2008) EphB receptors couple dendritic filopodia motility to synapse formation. *Neuron* 59:56–69.
49. Sieg DJ, et al. (2000) FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* 2:249–256.
50. Schaller MD, Otey CA, Hildebrand JD, Parsons JT (1995) Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J Cell Biol* 130: 1181–1187.
51. Beggs HE, Baragona SC, Hemperly JJ, Maness PF (1997) NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). *J Biol Chem* 272:8310–8319.
52. Miao H, Burnett E, Kinch M, Simon E, Wang B (2000) Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol* 2:62–69.
53. Dunty JM, et al. (2004) FERM domain interaction promotes FAK signaling. *Mol Cell Biol* 24:5353–5368.
54. Rico B, et al. (2004) Control of axonal branching and synapse formation by focal adhesion kinase. *Nat Neurosci* 7:1059–1069.
55. Phelan P, Gordon-Weeks R (1997) *Isolation of Synaptosomes, Growth Cones, and Their Subcellular Components* (Oxford University Press, Oxford), pp 1–38.
56. Biederer T, Scheiffele P (2007) Mixed-culture assays for analyzing neuronal synapse formation. *Nat Protoc* 2:670–676.