Essential cooperation of N-cadherin and neuroligin-1 in the transsynaptic control of vesicle accumulation

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Cell adhesion molecules are key players in transsynaptic communication, precisely coordinating presynaptic differentiation with postsynaptic specialization. At glutamatergic synapses, their retrograde signaling has been proposed to control presynaptic vesicle clustering at active zones. However, how the different types of cell adhesion molecules act together during this decisive step of synapse maturation is largely unexplored. Using a knockout approach, we show that two synaptic adhesion systems, N-cadherin and neuroligin-1, cooperate to control vesicle clustering at nascent synapses. Live cell imaging and fluorescence recovery after photobleaching experiments at individual synaptic boutons revealed a strong impairment of vesicle accumulation in the absence of N-cadherin, whereas the formation of active zones was largely unaffected. Strikingly, also the clustering of synaptic vesicles triggered by neuroligin-1 overexpression required the presence of N-cadherin in cultured neurons. Mechanistically, we found that N-cadherin acts by postsynaptically accumulating neuroligin-1 and activating its function via the scaffolding molecule S-SCAM, leading, in turn, to presynaptic vesicle clustering. A similar cooperation of N-cadherin and neuroligin-1 was observed in immature CA3 pyramidal neurons in an organotypic hippocampal network. Moreover, at mature synapses, N-cadherin was required for the increase in release probability and miniature EPSC frequency induced by expressed neuroligin-1. This cooperation of two cell adhesion systems provides a mechanism for coupling bidirectional synapse maturation mediated by neuroligin-1 to cell type recognition processes mediated by classical cadherins.

synapse maturation | synaptic adhesion molecules | vesicle clustering

Presynaptic accumulation of transmitter-filled vesicles is a hallmark process in the functional maturation of glutamatergic synapses in the CNS (1). The yet relatively unexplored molecular control mechanisms involved in vesicle clustering have been hypothesized to use retrograde signaling via transsynaptic adhesion molecules (2–6). Transsynaptic adhesion/signaling systems are based either on a homophilic or on a heterophilic protein–protein interaction across the synaptic cleft (7).

A prototypical example for heterophilically interacting transsynaptic proteins is the neuroligin/neurexin system (2, 6). In vitro expression of neuroligin-1 in a target cell induces presynaptic vesicle clusters in contacting axons (3, 8–10). Analysis of knockout mice established that neuroligins are important in functional maturation and validation of nascent synapses in vivo (11–13). However, because the neuroligin/neurexin system acts at various types of central synapses, the formation of specific synaptic connections within microcircuits appears to require additional mechanisms.

The homophilically interacting classical cadherins, such as N-cadherin, associate intracellularly with several types of catenins, thus forming the N-cadherin/catenin complex (14, 15). In addition to its important postsynaptic roles (4, 16–18), N-cadherin has been suggested to be involved in the formation of presynaptic vesicle clusters (4, 16, 19, 20). However, these findings could not yet be confirmed in N-cadherin knockout neurons (21, 22), and expression of N-cadherin in nonneuronal cells does not induce clustering of synaptic vesicles in contacting axons (3, 9). This observation emphasizes that the molecular mechanisms involved in the control of vesicle accumulation by N-cadherin are rather incompletely understood. Here, we present evidence that a cooperation of N-cadherin and neuroligin-1 via the postsynaptic scaffolding web is required to transsynaptically induce presynaptic vesicle clustering.

Results

Synaptic Vesicle Clustering Is Impaired in the Absence of N-Cadherin in Immature Neurons. We investigated presynaptic vesicle accumulation in cultured N-cadherin knockout neurons differentiated from homozygous N-cadherin knockout ES cells (21, 23; N-cad$^{-/-}$). The growth of axons and dendrites was not altered in N-cad$^{-/-}$ neurons (Fig. 1 *A*–*C*). In immature N-cad$^{-/-}$ neurons at 6–7 d in vitro (DIV), coimmunostaining for the synaptic vesicle protein synaptobrevin2 (VAMP2) and for the dendritic marker MAP2 revealed a significantly reduced density of presynaptic vesicle clusters on dendrites as compared with N-cad$^{+/-}$ controls (Fig. 1 *D* and *E*). In addition, the mean fluorescence intensity and the mean area of VAMP2 puncta on dendrites were significantly decreased in the absence of N-cadherin (Fig. 1 *F* and *G*). This reflected a specific defect in the accumulation of synaptic vesicles at active zones, because the density of clusters of the active zone protein Bassoon on dendrites was not changed in N-cad$^{-/-}$ neurons (Fig. 1 *H* and *I*). In more mature neurons at 10–14 DIV, vesicle clusters and Bassoon clusters were not significantly affected in N-cad$^{-/-}$ neurons, indicating compensatory mechanisms (Fig. S1). These results strongly suggest that the clustering of synaptic vesicles at active zones is specifically impaired in the absence of N-cadherin at nascent synapses.

To analyze vesicle accumulation during synapse maturation more directly, we performed live imaging upon coexpression of EGFP to visualize dendrites and of DsRed2-synaptobrevin2 (VAMP2) to visualize presynaptic vesicle clusters at autapses, which again showed a reduced density in N-cad$^{-/-}$ neurons at 6–7 DIV (Fig. S2). Time-lapse analysis of individual DsRed2-VAMP2 puncta revealed an impairment in the continuous increase in total DsRed2 fluorescence intensity in N-cad$^{-/-}$ neurons at 6–7 DIV (Fig. S3), indicating that ongoing vesicle accumulation at nascent synapses strongly depends on the expression of N-cadherin. In addition, an increase in the frequency of splitting/budding events

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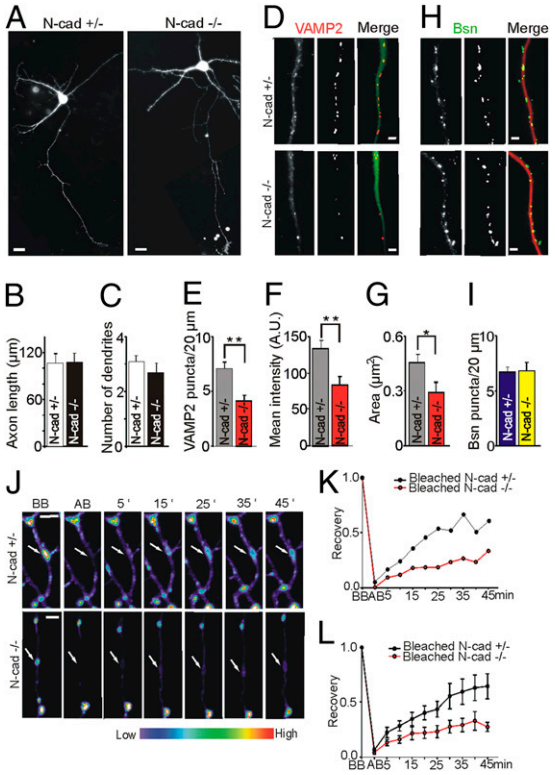

Fig. 1. Presynaptic vesicle accumulation depends on N-cadherin in immature neurons. (*A*) EGFP expressing mouse ES cell-derived neurons at 5 DIV (transfected at 3 DIV). N-cad$^{-/-}$, N-cadherin knockout neurons; N-cad$^{+/-}$, control neurons. (Scale bars: 10 μm.) (*B* and *C*) No change in axon length or in the number of dendrites was observed in N-cad$^{-/-}$ neurons. N-cad$^{+/-}$, $n = 30$; N-cad$^{-/-}$, $n = 30$. (*D* and *H*) Immunocytochemical stainings of vesicle clusters (VAMP2) and Bassoon clusters (Bsn) on dendrites at 6–7 DIV. (*Right*) Original images. (*Center*) Thresholded VAMP2 or Bsn puncta. (*Left*) Overlay image of VAMP2 or Bsn puncta and MAP2 staining of dendrites. (Scale bars: 2 μm.) (*E–G* and *I*) Dendritic density (*E*), mean intensity (*F*), and area (*G*) of VAMP2 puncta are reduced in the absence of N-cadherin, whereas clusters of the active zone protein Bassoon are unaltered (*I*). VAMP2 puncta: N-cad$^{+/-}$, $n = 20$; N-cad$^{-/-}$, $n = 19$; Bsn puncta: N-cad$^{+/-}$, $n = 21$; N-cad$^{-/-}$, $n = 16$. Mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$, unpaired t test. (*J*) Examples of FRAP experiments in EGFP-VAMP2 expressing N-cad$^{+/-}$ and N-cad$^{-/-}$ neurons at 6–7 DIV (transfected at 4–5 DIV). Individual EGFP-VAMP2 puncta on dendrites were photobleached (arrows) and fluorescence recovery was imaged at indicated time points. Fluorescence intensity is color-coded. BB, before photobleaching; AB, after photobleaching ($t = 0$ min). (Scale bars: 5 μm.) (*K*) Normalized fluorescence intensity of photobleached vesicle clusters shown in *J* versus observation time. (*L*) Mean recovery of fluorescence intensity. N-cad$^{+/-}$, $n = 13$; N-cad$^{-/-}$, $n = 11$. Mean ± SEM.

of DsRed2-VAMP2 puncta was found during time-lapse recording in N-cad$^{-/-}$ neurons (Fig. S4), suggesting that N-cadherin is also important for maintaining vesicle cluster integrity. To study vesicle accumulation in a quantitatively controlled manner, we performed fluorescence recovery after photobleaching (FRAP) experiments at 6–7 DIV in EGFP-VAMP2 expressing N-cad$^{-/-}$ neurons. Individual EGFP-VAMP2 puncta on dendrites were locally photobleached by using laser light and then the recovery of EGFP fluorescence was monitored with live-imaging for 45 min (Fig. 1 *J–L*). In control N-cad$^{+/-}$ neurons, >60% recovery was reached after 45 min, whereas in N-cad$^{-/-}$ neurons, only ≈30% recovery was observed. This finding strongly confirms that the accumulation of vesicles is severely impaired in the absence of N-cadherin. In addition, alterations in vesicle trafficking might contribute to the reduced recovery.

Vesicle Cluster-Inducing Activity of Neuroligin-1 Requires Expression of N-Cadherin. Although N-cadherin plays an important role in vesicle clustering, the expression of N-cadherin in nonneuronal cells (3, 9) or the overexpression of N-cadherin in cultured cortical neurons (Fig. S5) did not lead to formation of additional vesicle clusters in contacting neurons. In contrast, neuroligin-1 in the same assay systems is a potent inducer of vesicle clusters (3, 8, 9). Thus, we hypothesized that N-cadherin might control presynaptic vesicle accumulation by recruiting and activating the neuroligin-1 system. To address this potential cooperation mechanism experimentally, we expressed neuroligin-1-EGFP in immature N-cad$^{-/-}$ neurons at 6–7 DIV. In N-cad$^{+/-}$ control neurons, coexpression of neuroligin-1-EGFP and DsRed2-VAMP2 led to a highly significant increase in the density of vesicle clusters on dendrites (148 ± 8%). Strikingly, this vesicle cluster-inducing effect of neuroligin-1 was completely absent upon expression of neuroligin-1-EGFP in N-cad$^{-/-}$ neurons (Fig. 2 *A–C*). This finding strongly indicates that in neurons, N-cadherin is required to enable neuroligin-1 to exert its vesicle cluster-inducing effect.

Next, we studied the subcellular localization of the expressed neuroligin-1-EGFP along dendrites. In the absence of N-cadherin, the density of neuroligin-1-EGFP puncta was significantly reduced (Fig. S5), suggesting that N-cadherin is required for clustering of neuroligin-1. In addition, the fraction of neuroligin-1-EGFP puncta colocalized with DsRed2-VAMP2 puncta (autapses) was strongly reduced in Ncad$^{-/-}$ neurons (Fig. S5). We further confirmed our findings with expressed proteins by examining the endogenous neuroligin distribution in N-cad$^{-/-}$ neurons at 6–7 DIV by using immunostaining. In the absence of N-cadherin, the density of endogenous neuroligin clusters was strongly reduced (Fig. 2 *D–F*). Furthermore, the fraction of endogenous neuroligin clusters apposed to coimmunostained VAMP2 puncta was strongly diminished. We also coimmunostained endogeneous neuroligin and PSD95 clusters. In the absence of N-cadherin, the density of PSD95 puncta was reduced in parallel with the loss of neuroligin clusters (Fig. 2*G* and Fig. S6). This finding is in line with a role of neuroligin-1 in synaptic recruitment of PSD95 (24). Neuroligin puncta partially colocalized with PSD95 puncta (Fig. S6), indicating the presence of both relatively mature and nascent synaptic contacts. In summary, our findings strongly suggest a functional cooperation of the N-cadherin/catenin and the neuroligin-1 adhesion systems. This cooperation ensures the proper postsynaptic localization of neuroligin-1 clusters apposed to presynaptic specializations during early synapse maturation.

Functional Cooperation of N-Cadherin and Neuroligin-1 Depends on the Postsynaptic Scaffolding Web. Mechanistically, functional cooperation might be mediated by postsynaptic connecting molecules interacting with both neuroligin-1 and N-cadherin. Postsynaptic scaffolding proteins binding to neuroligin-1 such as S-SCAM (MAGI-2) have been hypothesized to be able to link the two adhesion systems via binding to δ-catenin or β-catenin (25–29). To examine the potential role of S-SCAM, we expressed truncated forms of S-SCAM that were designed to inhibit protein interactions of S-SCAM in a dominant-negative manner. For inhibition of the interaction of endogeneous S-SCAM with neuroligin-1, we expressed a mutant S-SCAM lacking the PDZ5 domain (binding to β-catenin/N-cadherin). For inhibition of the interaction of N-cadherin/β-catenin with endogeneous S-SCAM, we expressed a mutant S-SCAM lacking the WW and PDZ1 domains (binding to neuroligin-1).

S-SCAM proteins were coexpressed together with neuroligin-1-EGFP and DsRed2-VAMP2 in cultured cortical neurons. Expressed EGFP-S-SCAM was present in the dendrites in a largely homogeneous distribution (Fig. S7). The control coexpression of full-length S-SCAM resulted in a clear vesicle cluster-inducing effect of neuroligin-1 (152 ± 12% of EGFP expressing controls; Fig. 3 *A* and *B*). Coexpression of either of the two mutant S-SCAM proteins strongly inhibited the vesicle cluster-inducing activity of expressed

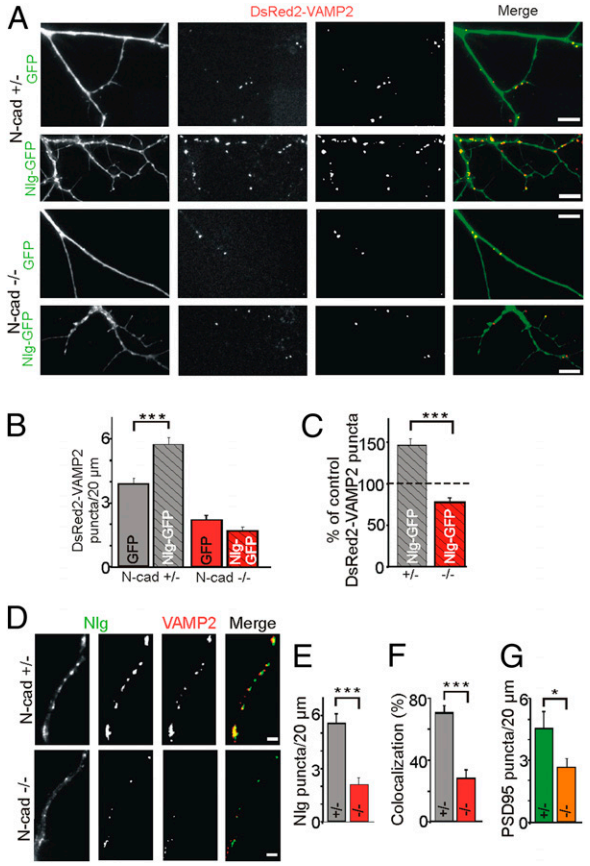

Fig. 2. N-cadherin is required for both the induction of presynaptic vesicle clusters by neuroligin-1 and the postsynaptic clustering of neuroligin. (*A*) Images of dendrites of N-cad$^{+/-}$ and N-cad$^{-/-}$ neurons (6-7 DIV) cotransfected (at 4–5 DIV) with DsRed2-VAMP2 and either neuroligin-1-EGFP (Nlg-GFP) or EGFP. (*Left*) EGFP-labeled dendrites. (*Center*) DsRed2-VAMP2-labeled vesicle clusters (*Center Left*, original images; *Center Right*, thresholded DsRed2-VAMP2 puncta). (*Right*) Overlay of EGFP-labeled dendrites (green) and DsRed2-VAMP2 puncta (red). (Scale bars: 5 µm.) (*B* and *C*) Quantification of the induction of presynaptic vesicle clusters by overexpression of neuroligin-1. Coexpression of either neuroligin-1-EGFP or EGFP with DsRed2-VAMP2 is indicated on bars. N-cad$^{+/-}$: GFP, n = 45; Nlg-GFP, n = 31. N-cad$^{-/-}$: GFP, n = 38; Nlg-GFP, n = 36. (*D*) Immunostainings for endogenous neuroligin (Nlg) and VAMP2 in N-cad$^{+/-}$ and N-cad$^{-/-}$ neurons at 7 DIV. (*D Left*) Original images of endogenous neuroligin and corresponding thresholded neuroligin puncta. (*Center*) Thresholded VAMP2 puncta. (*Right*) Overlay of thresholded neuroligin (green) and VAMP2 puncta (red) visualizing colocalization. (Scale bars: 2 µm.) (*E*) Density of endogenous neuroligin puncta in dendrites. N-cad$^{+/-}$, n = 19; N-cad$^{-/-}$, n = 21. (*F*) Colocalization of neuroligin puncta with VAMP2 puncta. N-cad$^{+/-}$, n = 22; N-cad$^{-/-}$, n = 15. (*G*) Density of endogeneous PSD95 puncta in dendrites. N-cad$^{+/-}$, n = 21; N-cad$^{-/-}$, n = 21. Mean ± SEM; *P < 0.05; ***P < 0.001, unpaired t test.

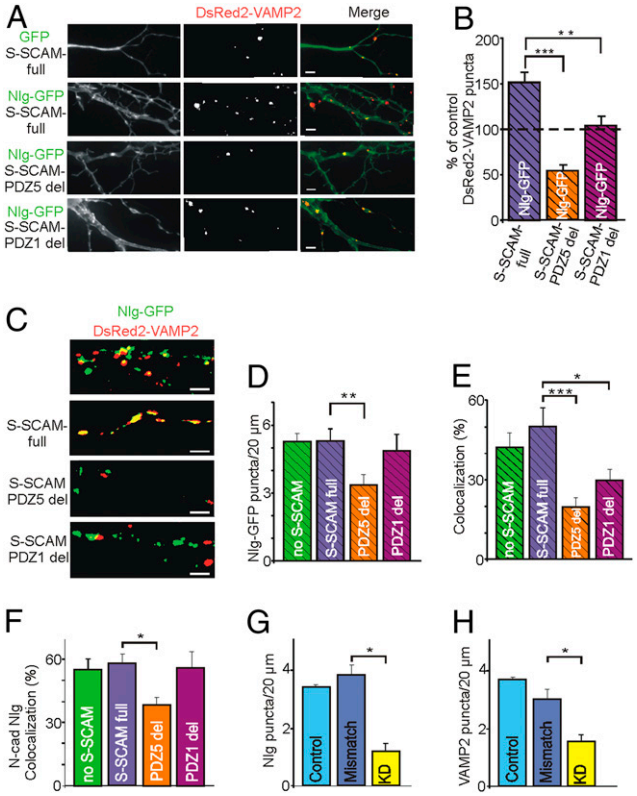

Fig. 3. Cooperation of N-cadherin and neuroligin-1 is mediated by the scaffolding protein S-SCAM in cultured cortical neurons. (*A* and *B*) Inhibition of S-SCAM function by expression of truncated S-SCAM proteins blocks the induction of presynaptic vesicle clusters by neuroligin-1-EGFP (transfected at 4–5 DIV, analyzed at 6–7 DIV). (*A*) DsRed2-VAMP2 was coexpressed with either EGFP or neuroligin-1-EGFP (Nlg-GFP) and either full-length S-SCAM (S-SCAM full) or S-SCAM with PDZ5 deleted (S-SCAM-PDZ5 del) or S-SCAM with WW and PDZ1 deleted (S-SCAM-PDZ1 del). (*Left*) EGFP-labeled dendrites. (*Center*) Thresholded DsRed2-VAMP2 puncta. (*Right*) Overlay of EGFP fluorescence (green) and DsRed2-VAMP2 puncta (red) to visualize vesicle clusters on dendrites. (Scale bars: 2 µm.) (*B*) Changes in vesicle cluster density upon neuroligin-1-EGFP expression. Type of coexpressed S-SCAM is indicated below bars. S-SCAM-full, n = 16; S-SCAM-PDZ5del, n = 20; S-SCAM-PDZ1del, n = 17. (*C–E*) Clustering of neuroligin-1-EGFP and colocalization with vesicle clusters depends on S-SCAM function. (*C*) Overlay images of thresholded neuroligin-1-EGFP puncta (green) and DsRed2-VAMP2 puncta (red) to visualize colocalization. Type of coexpressed S-SCAM is indicated. (Scale bar, 2 µm.) Density of neuroligin-1-EGFP puncta on dendrites (*D*), and colocalization of neuroligin-1-EGFP puncta with DsRed2-VAMP2 puncta (*E*). No S-SCAM, n = 27; S-SCAM full, n = 16; S-SCAM PDZ5del, n = 20; S-SCAM PDZ1del, n = 17. (*F*) Colocalization of immunoctochemically stained endogeneous N-cadherin puncta with endogeneous neuroligin puncta depends on S-SCAM function. No S-SCAM, n = 13; S-SCAM full, n = 11; S-SCAM PDZ5del, n = 12; S-SCAM PDZ1del, n = 13. (*G* and *H*) RNAi-mediated S-SCAM knockdown in low-density cultures of cortical neurons (transfected at 2 DIV, analyzed at 8 DIV). Quantification of immunocytochemically stained neuroligin puncta (*G*) and VAMP2 puncta (*H*) at 8 DIV. Control, no transfection; Mismatch, mismatch control; KD, knockdown. Mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA; unpaired t test in *G* and *H*.

neuroligin-1 (Fig. 3 *A* and *B*), suggesting that interactions of endogeneous S-SCAM with both neuroligin-1 and N-cadherin/β-catenin are essential for the induction of presynaptic vesicle clustering.

To address whether S-SCAM is in addition to functional activation also involved in postsynaptic clustering of the expressed neuroligin-1, we analyzed the density of neuroligin-1-EGFP puncta and their colocalization with presynaptic vesicle clusters. Inhibition of the S-SCAM neuroligin-1 interaction resulted in a significantly reduced density of neuroligin-1-EGFP puncta and in a significantly reduced colocalization of neuroligin-1-EGFP puncta with presynaptic vesicle clusters (Fig. 3 *C–E* and Fig. S7). Interestingly, inhibition of the β-catenin S-SCAM interaction did not result in a significant reduction of neuroligin-1-EGFP puncta but led to a significantly reduced colocalization of neuroligin-1-EGFP puncta with presynaptic vesicle clusters (Fig. 3 *C–E* and Fig. S7). These results suggest that N-cadherin is able to induce neuroligin-1 clustering by molecular pathways that involve the S-SCAM neuroligin-1 interaction, but appear not to require the N-cadherin/β-catenin/S-SCAM interaction. However, functional activation of neuroligin-1 leading to presynaptic vesicle accumulation requires the N-cadherin/β-catenin/S-SCAM pathway.

To further investigate the differential effects of the two truncated forms of S-SCAM on neuroligin clustering, we analyzed the

colocalization of endogeneous N-cadherin and endogeneous neuroligin in cultured cortical neurons (6-7 DIV) by using immunocytochemistry. Expression of S-SCAM constructs did not affect the density of N-cadherin puncta (Fig. S8). Inhibition of the S-SCAM neuroligin-1 interaction again resulted in a significantly reduced density of neuroligin puncta and in a significantly reduced colocalization of N-cadherin puncta with neuroligin puncta (Fig. 3*F* and Fig. S8). In contrast, inhibition of the β-catenin S-SCAM interaction did not result in a significant reduction of neuroligin puncta and did not affect colocalization of N-cadherin puncta with neuroligin puncta. In summary, our results with expression of truncated forms of S-SCAM strongly indicate that the binding of endogeneous S-SCAM to neuroligin-1 is important in the control of neuroligin-1 clustering by N-cadherin and in the functional activation of neuroligin-1 leading to presynaptic vesicle clustering. Neuroligin-1 can still be clustered if the β-catenin interaction with endogeneous S-SCAM is inhibited, but under these conditions, the functional activation of neuroligin-1 is blocked.

To further study the role of S-SCAM, we performed an RNAi-mediated knockdown of all S-SCAM isoforms by transfecting cultured cortical neurons with shRNA lentiviral vectors (Fig. S9). Effects of S-SCAM knockdown were analyzed at 8 DIV by immuncytochemical stainings for neuroligin, VAMP2, and N-cadherin. We observed a significant reduction in both the dendritic density of neuroligin puncta and the density of VAMP2 puncta on dendrites (Fig. 3 *G* and *H*), confirming that S-SCAM is involved in the clustering of neuroligin and in its vesicle cluster-inducing activity. In addition, the density of N-cadherin puncta was reduced (Fig. S9), indicating an interdependence of N-cadherin and S-SCAM. In summary, our results demonstrate that S-SCAM is crucial for the cooperation between the N-cadherin/catenin and the neuroligin-1 systems, leading to a transsynaptic regulation of presynaptic maturation.

Functional Cooperation of N-Cadherin and Neuroligin-1 in CA3 Pyramidal Neurons Within an Organotypic Hippocampal Network. We further wanted to examine whether the cooperation of N-cadherin and neuroligin-1 is also important in neurons within an organotypic neuronal network. Because N-cadherin knockout mice are lethal at an early embryonic stage (30) and conditional N-cadherin knockout leads to largely disorganized neuronal networks (22), we inhibited N-cadherin function by postsynaptically expressing a dominant-negative N-terminally truncated form of N-cadherin lacking the extracellular cadherin domains (N-cadΔE; ref. 31). Expression of N-cadΔE has been shown to result in a strong mislocalization of β-catenin (20), thus resulting in a severe block of N-cadherin function comparable with knockout experiments. Because it interferes with catenin binding to the C-terminal domain of all classical cadherins, N-cadΔE might affect the function of other cadherins in addition to N-cadherin. CA3 pyramidal neurons in organotypic slice cultures of rat hippocampus were cotransfected with N-cadΔE and neuroligin-1-EGFP by using single-cell electroporation. After 3D reconstruction of fluorescent proximal dendrites, the density of coimmunostained VAMP2 puncta on dendrites was determined at 7 DIV (Fig. 4 *A–D*). At this relatively early stage of development in slice culture, CA3 pyramidal neurons exhibited only few spines. As expected, expression of neuroligin-1-EGFP resulted in a significantly increased density of presynaptic vesicle clusters (154 ± 13%). Coexpression of N-cadΔE completely inhibited the vesicle cluster-inducing activity of expressed neuroligin-1, strongly confirming that N-cadherin controls the functional activation of neuroligin-1. Because neuroligin-1 immunohistochemistry in brain slices is impeded by the lack of antibodies suitable for staining in brain tissue, we studied neuroligin-1 clustering by analyzing the density of dendritic neuroligin-1-EGFP puncta. We found a significantly decreased density of neuroligin-1-EGFP puncta in N-cadΔE expressing CA3 pyramidal neurons (Fig. 4 *E* and *F*), indicating that N-cadherin function

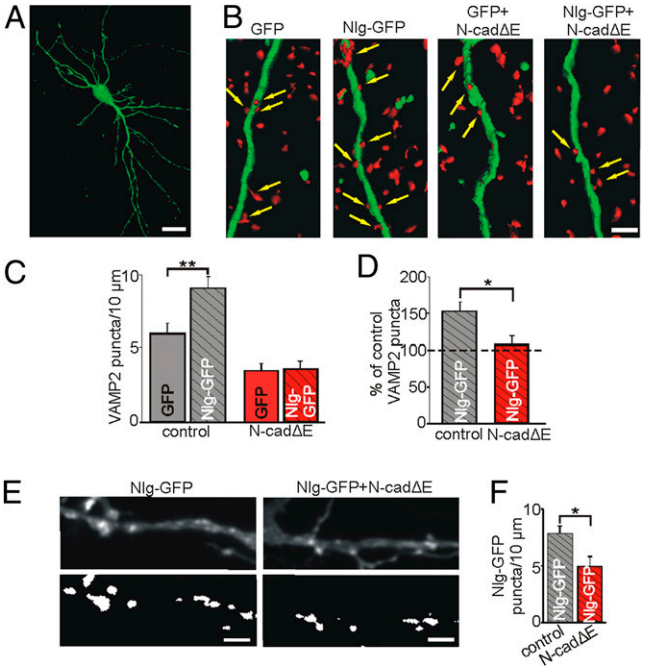

Fig. 4. Cooperation of N-cadherin and neuroligin-1 in CA3 pyramidal neurons. (*A*) Three-dimensional reconstruction of an EGFP expressing CA3 pyramidal neuron in a rat hippocampal slice culture (transfected by single cell electroporation). (Scale bar, 10 μm.) (*B–D*) Neuroligin-1-EGFP (Nlg-GFP) or EGFP were coelectroporated with dominant-negative N-cadΔE (ectodomain deleted) into single cells at 5 DIV and presynaptic vesicle clusters were immunostained for VAMP2 2 d later at 7 DIV. (*B*) Three-dimensional images of proximal dendrites. VAMP2 puncta on EGFP expressing dendrites are indicated by arrows. (Scale bar, 3 μm.) (*C*) Density of VAMP2 puncta on dendrites. (*D*) Change in VAMP2 puncta density upon neuroligin-1-EGFP expression depends on N-cadherin function. (*E* and *F*) Neuroligin-1-EGFP clustering depends on N-cadherin function. (*E*) (*Upper*) Original images of neuroligin-1-EGFP-expressing dendrites. (*Lower*) Thresholded neuroligin-1-EGFP puncta. (Scale bars: 2 μm.) (*F*) Dendritic density of neuroligin-1-EGFP puncta. Mean ± SEM; $*P < 0.05$; $**P < 0.01$, unpaired *t* test.

is important for neuroligin-1 clustering. In summary, these results strongly confirm our findings in cultured ES cell-derived neurons and in cultured cortical neurons in an organotypic neuronal network.

Neuroligin-1 Requires N-Cadherin for Increasing Miniature EPSC Frequency and Release Probability at Mature Synapses. In addition to its vesicle-cluster-inducing activity, neuroligin-1 overexpression has been shown to increase AMPA receptor-mediated mEPSC frequency in cultured neurons (8, 32). To examine whether the neuroligin-1-induced increase in miniature frequency also requires the expression of N-cadherin, we performed whole-cell patch-clamp recordings in neuroligin-1 overexpressing N-cad$^{-/-}$ neurons at 12–14 DIV. In control N-cad$^{+/-}$ neurons, the mean frequency of AMPA mEPSCs significantly increased 3-fold upon expression of neuroligin-1-EGFP. Strikingly, in N-cad$^{-/-}$ neurons, the mean mEPSC frequency did not significantly increase upon neuroligin-1-EGFP overexpression (Fig. 5 *A–C*), indicating that neuroligin-1 cooperates with N-cadherin for exerting its effect on miniature frequency. No changes in mEPSC amplitudes were observed. Furthermore, overexpression of neuroligin-1 has been described to increase release probability in hippocampal neurons (33). We studied the effects of neuroligin-1 on release probability by analyzing the progressive block of evoked NMDA EPSCs by MK-801 (20 μM). We used cultured cortical neurons at 12–14 DIV, because NMDA EPSCs in ES cell-derived neurons exhibit only very small amplitudes (21). With N-cadherin function impaired by N-cadΔE coexpression,

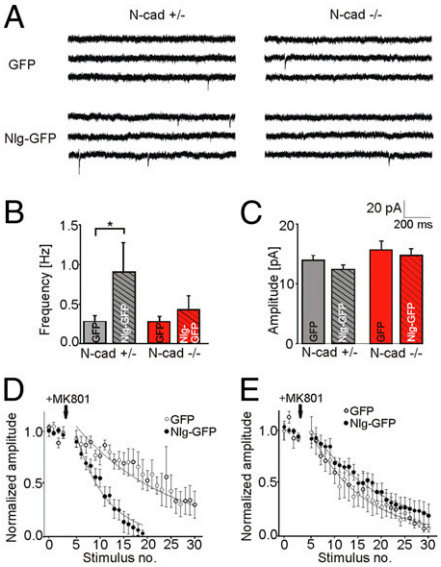

Fig. 5. Neuroligin-1-induced increase in both miniature EPSC frequency and release probability requires N-cadherin function. (*A*) Example traces of whole-cell recordings of AMPA mEPSCs in N-cad$^{-/-}$ and control (N-cad$^{+/-}$) neurons at 12–14 DIV expressing either neuroligin-1-EGFP (Nlg-GFP) or EGFP (transfected at 10–12 DIV). Holding potential: −60 mV. Frequencies (*B*) and amplitudes (*C*) of AMPA mEPSCs upon neuroligin-1-EGFP overexpression. N-cad$^{+/-}$: GFP, $n = 35$; Nlg-GFP, $n = 24$. N-cad$^{-/-}$: GFP, $n = 28$; Nlg-GFP, $n = 25$. Mean ± SEM; $^*P < 0.05$, unpaired *t* test. (*D*) Neuroligin-1-EGFP overexpression ($n = 6$) accelerated the MK801 block of evoked NMDA EPSCs in cultured cortical neurons at 12–14 DIV (transfected at 10–12 DIV) as compared with control EGFP expression ($n = 5$), indicating an increased release probability. (*E*) Upon coexpression of N-cadΔE the enhancing effect of neuroligin-1 on release probability was blocked ($n = 5$; controls $n = 5$). Mean ± SEM. Biexponential fits are shown.

the enhancing effect of neuroligin-1 expression on release probability was blocked (Fig. 5 *D* and *E*). These findings indicate that the cooperation of N-cadherin and neuroligin-1 is also involved in the functional activation of neuroligin-1's vesicle release modulating activity, thus regulating synaptic function.

Discussion

Using a knockout approach selectively targeting N-cadherin, we show here that the accumulation of vesicles strongly depends on N-cadherin at nascent synapses. In agreement to our results, expression of a dominant-negative mutant N-cadherin led to a reduction in the number of vesicle clusters that was most pronounced in immature neurons (16, 20). In addition, β-catenin- (19) and p120catenin-deficient (17) hippocampal neurons showed a reduced clustering of presynaptic vesicles. At the neuromuscular junction, the adhesion molecule NCAM has been described to play an analogous role (34). Direct presynaptic catenin signaling via the actin cytoskeleton and via cytoplasmic proteins has been proposed to mediate the regulation of vesicle clustering by the N-cadherin/catenin system (17, 19, 35). However, a redistribution of β-catenin from dendritic shafts to spines resulted in an increase in presynaptic synapsin-I cluster size and signal intensity (4), suggesting the involvement of retrograde signaling pathways. In this paper, we provide evidence for a transsynaptic retrograde mechanism controlling presynaptic vesicle accumulation that is based on a postsynaptic cooperation between N-cadherin and neuroligin-1. We found a strict dependence of neuroligin-1's vesicle cluster-inducing activity on the expression of N-cadherin. Moreover, our analysis of the subcellular localization of a neuroligin-1-EGFP fusion protein and of endogenous neuroligin indicated an impairment of the synaptic targeting of neuroligin-1 in the absence of N-cadherin. These results were confirmed in CA3 pyramidal neurons in an organotypic hippocampal network by using expression of a dominant-negative N-cadherin mutant protein that induces mislocalization of catenins (20). In line with our results on the cooperation of the N-cadherin/catenin and the neuroligin-1 adhesion systems, recent studies in neuroligin-1 knockout mice and in neuroligin-1 overexpressing mice demonstrated an in vivo role of neuroligin-1 in glutamatergic synapse maturation including presynaptic vesicle clustering (12, 13, 36).

Postsynaptic clustering of ion channels and adhesion molecules is controlled by scaffolding proteins (37, 38), and overexpression of postsynaptic scaffolding proteins retrogradely enhances maturation of presynaptic terminals (5, 39). The MAGI type postsynaptic scaffolding protein S-SCAM (26, 29) has been described to potentially interact with catenins via binding with its PDZ5 domain to the PDZ-binding domains of β-catenin and δ-catenin (28, 40). Furthermore, S-SCAM has been suggested to bind with its WW and PDZ1 domain to neuroligin-1 (27). However, a functional role at the synapse for the potential interaction between S-SCAM and neuroligin-1 could not be demonstrated (27). Here, we show that expression of mutant S-SCAM proteins and S-SCAM knockdown lead to a functional block of the cooperation between the N-cadherin/catenin and the neuroligin-1 systems and, as a result, inhibit presynaptic vesicle accumulation. These findings strongly suggest that the postsynaptic scaffolding web functionally connects the N-cadherin/catenin and the neuroligin-1 adhesion systems.

N-cadherin appeared to regulate synaptic targeting of neuroligin-1 via postsynaptic scaffolding molecules. Both the number of postsynaptic neuroligin-1 clusters and their colocalization with presynaptic vesicles were reduced in the absence of N-cadherin, upon inhibition of the S-SCAM neuroligin-1 interaction, and upon S-SCAM knockdown. We further demonstrated that inhibition of the S-SCAM neuroligin-1 interaction led to a reduced colocalization of N-cadherin puncta with Neuroligin clusters, strongly indicating that N-cadherin controls neuroligin-1 targeting via S-SCAM. In line with this mechanism, it has been suggested that synaptic targeting of neuroligin-1 is independent of PSD95 binding (41). Interestingly, in mature cultured hippocampal neurons exhibiting spines, synaptic targeting of neuroligin-1 did not require the C-terminal PDZ domain binding motif of neuroligin-1 (41), indicating alternative mechanisms. GPI-neuroligin-1-coated beads are sufficient to induce vesicle accumulation in hippocampal neurons (42). However, the density of GPI-neuroligin-1 molecules on these beads appears to be rather high, which renders the physiological mechanisms for neuroligin-1 clustering dispensable in this artifical situation.

In addition to synaptic targeting, a functional activation of neuroligin-1 by scaffolding proteins appeared to be of crucial importance. Selective inhibition of the β-catenin S-SCAM interaction did not lead to altered neuroligin-1 clustering, indicating that effects on clustering might be compensated by other catenins still binding to N-cadherin. However, the colocalization of neuroligin-1 clusters with presynaptic vesicles was strongly reduced indicating a block of neuroligin-1's vesicle cluster-inducing activity. In summary, our results demonstrate that the postsynaptic scaffolding web is required to link the N-cadherin/catenin and the neuroligin-1 systems both structurally and functionally. In our scenario, the involvement of N-cadherin is not exclusively postsynaptic. Presynaptic N-cadherin would also be a decisive molecular player, because it might determine the axonal location where vesicles are accumulated by homophilic transsynaptic binding to postsynaptic N-cadherin.

The molecular cooperation between the N-cadherin/catenin and the neuroligin-1 adhesion systems might be of importance in the selective validation of nascent synapses, thus contributing to synapse specificity. Classical cadherins including N-cadherin have been proposed to play a central role in specific neuronal recognition processes because of their homophilic binding (7, 14, 15).

Neuroligins on the other hand have been shown to play a crucial role in validating immature synapses (10, 12, 13). This function involves heterophilic interactions with neurexins (6), but may also be mediated by neurexin-independent transsynaptic signaling (10). Upon overexpression of neuroligin-1 in cultured hippocampal neurons, the number of presynaptic synapsin puncta was increased independent of neurexin binding, whereas the increase in the size of presynaptic synapsin puncta depended on binding to α-neurexin (10). The cooperation between the N-cadherin and neuroligin-1 adhesion systems couples their functional roles and thus might enable a selective validation and maturation of matching synapses between neurons recognizing each other via cadherins. This molecular mechanism thus might link specific target recognition to bidirectional signaling required for synapse differentiation.

Methods

In vitro differentiation, immunoisolation, and cultivation of mouse ES cell-derived, N-cadherin knockout neurons were performed as described (21, 43), except that neurons were cultured on a confluent glial monolayer resulting in the formation of both synapses and autapses. Mouse cortical neurons and rat hippocampal slices were cultured according to standard protocols. Fluorescence imaging of cultured neurons was done by obtaining *z* stacks of fluorescence images by using a computer-controlled, motorized Zeiss Axiovert 200M inverted microscope enabling 3D deconvolution of the obtained images. Image acquisition was done with a 12-bit monochrome CoolSNAP ES CCD camera (Photometrics) by using MetaView software (Visitron Systems). Upon 3D deconvolution, images were analyzed by using MetaMorph software. FRAP experiments and fluorescence imaging in fixed hippocampal slice cultures were performed by using a confocal laser scanning microscope (Zeiss LSM 510). RNAi-mediated S-SCAM knockdown was performed by using lentiviral infection of cortical neurons in low-density cultures. Transfection in slice cultures was done by single-cell electroporation. Immunocytochemistry and electrophysiology were performed by using standard conditions. All protocols are described thoroughly in *SI Methods*.

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1. Garner CC, Waites CL, Ziv NE (2006) Synapse development: Still looking for the forest, still lost in the trees. *Cell Tissue Res* 326:249–262.
2. Ichtchenko K, et al. (1995) Neuroligin 1: A splice site-specific ligand for beta-neurexins. *Cell* 81:435–443.
3. Scheiffele P, Fan J, Choih J, Fetter R, Serafini T (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101:657–669.
4. Murase S, Mosser E, Schuman EM (2002) Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* 35:91–105.
5. Regalado MP, Terry-Lorenzo RT, Waites CL, Garner CC, Malenka RC (2006) Transsynaptic signaling by postsynaptic synapse-associated protein 97. *J Neurosci* 26:2343–2357.
6. Südhof TC (2008) Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* 455:903–911.
7. Yamada S, Nelson WJ (2007) Synapses: Sites of cell recognition, adhesion, and functional specification. *Annu Rev Biochem* 76:267–294.
8. Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. *Proc Natl Acad Sci USA* 101:13915–13920.
9. Sara Y, et al. (2005) Selective capability of SynCAM and neuroligin for functional synapse assembly. *J Neurosci* 25:260–270.
10. Ko J, et al. (2009) Neuroligin-1 performs neurexin-dependent and neurexin-independent functions in synapse validation. *EMBO J* 28:3244–3255.
11. Varoqueaux F, et al. (2006) Neuroligins determine synapse maturation and function. *Neuron* 51:741–754.
12. Chubykin AA, et al. (2007) Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. *Neuron* 54:919–931.
13. Wittenmayer N, et al. (2009) Postsynaptic Neuroligin1 regulates presynaptic maturation. *Proc Natl Acad Sci USA* 106:13564–13569.
14. Benson DL, Colman DR, Huntley GW (2001) Molecules, maps and synapse specificity. *Nat Rev Neurosci* 2:899–909.
15. Takeichi M (2007) The cadherin superfamily in neuronal connections and interactions. *Nat Rev Neurosci* 8:11–20.
16. Togashi H, et al. (2002) Cadherin regulates dendritic spine morphogenesis. *Neuron* 35: 77–89.
17. Elia LP, Yamamoto M, Zang K, Reichardt LF (2006) p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins. *Neuron* 51:43–56.
18. Tai CY, Kim SA, Schuman EM (2008) Cadherins and synaptic plasticity. *Curr Opin Cell Biol* 20:567–575.
19. Bamji SX, et al. (2003) Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. *Neuron* 40:719–731.
20. Bozdagi O, Valcin M, Poskanzer K, Tanaka H, Benson DL (2004) Temporally distinct demands for classic cadherins in synapse formation and maturation. *Mol Cell Neurosci* 27:509–521.
21. Jüngling K, et al. (2006) N-cadherin transsynaptically regulates short-term plasticity at glutamatergic synapses in embryonic stem cell-derived neurons. *J Neurosci* 26: 6968–6978.
22. Kadowaki M, et al. (2007) N-cadherin mediates cortical organization in the mouse brain. *Dev Biol* 304:22–33.
23. Moore R, Radice GL, Dominis M, Kemler R (1999) The generation and in vivo differentiation of murine embryonal stem cells genetically null for either N-cadherin or N- and P-cadherin. *Int J Dev Biol* 43:831–834.
24. Barrow SL, et al. (2009) Neuroligin1: A cell adhesion molecule that recruits PSD-95 and NMDA receptors by distinct mechanisms during synaptogenesis. *Neural Develop* 4:17.
25. Irie M, et al. (1997) Binding of neuroligins to PSD-95. *Science* 277:1511–1515.
26. Hirao K, et al. (1998) A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. *J Biol Chem* 273:21105–21110.
27. Iida J, Hirabayashi S, Sato Y, Hata Y (2004) Synaptic scaffolding molecule is involved in the synaptic clustering of neuroligin. *Mol Cell Neurosci* 27:497–508.
28. Nishimura W, Yao I, Iida J, Tanaka N, Hata Y (2002) Interaction of synaptic scaffolding molecule and beta-catenin. *J Neurosci* 22:757–765.
29. Deng F, Price MG, Davis CF, Mori M, Burgess DL (2006) Stargazin and other transmembrane AMPA receptor regulating proteins interact with synaptic scaffolding protein MAGI-2 in brain. *J Neurosci* 26:7875–7884.
30. Radice GL, et al. (1997) Developmental defects in mouse embryos lacking N-cadherin. *Dev Biol* 181:64–78.
31. Riehl R, et al. (1996) Cadherin function is required for axon outgrowth in retinal ganglion cells in vivo. *Neuron* 17:837–848.
32. Levinson JN, et al. (2005) Neuroligins mediate excitatory and inhibitory synapse formation: Involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem* 280:17312–17319.
33. Futai K, et al. (2007) Retrograde modulation of presynaptic release probability through signaling mediated by PSD-95-neuroligin. *Nat Neurosci* 10:186–195.
34. Rafuse VF, Polo-Parada L, Landmesser LT (2000) Structural and functional alterations of neuromuscular junctions in NCAM-deficient mice. *J Neurosci* 20:6529–6539.
35. Zhang W, Benson DL (2001) Stages of synapse development defined by dependence on F-actin. *J Neurosci* 21:5169–5181.
36. Dahlhaus R, et al. (2010) Overexpression of the cell adhesion protein neuroligin-1 induces learning deficits and impairs synaptic plasticity by altering the ratio of excitation to inhibition in the hippocampus. *Hippocampus* 20:305–322.
37. Heine M, et al. (2008) Activity-independent and subunit-specific recruitment of functional AMPA receptors at neurexin/neuroligin contacts. *Proc Natl Acad Sci USA* 105: 20947–20952.
38. Nam CI, Chen L (2005) Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc Natl Acad Sci USA* 102:6137–6142.
39. Sala C, et al. (2001) Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31:115–130.
40. Ide N, et al. (1999) Interaction of S-SCAM with neural plakophilin-related Armadillo-repeat protein/delta-catenin. *Biochem Biophys Res Commun* 256:456–461.
41. Dresbach T, Neeb A, Meyer G, Gundelfinger ED, Brose N (2004) Synaptic targeting of neuroligin is independent of neurexin and SAP90/PSD95 binding. *Mol Cell Neurosci* 27:227–235.
42. Dean C, et al. (2003) Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* 6:708–716.
43. Jüngling K, Nägler K, Pfrieger FW, Gottmann K (2003) Purification of embryonic stem cell-derived neurons by immunoisolation. *FASEB J* 17:2100–2102.