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Distinct roles of neuroligin-1 and SynCAM1 in synapse formation and function in primary hippocampal neuronal cultures

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Abstract

Neuroligins are a family of cell adhesion molecules critical in establishing proper central nervous system connectivity; disruption of neuroligin signaling *in vivo* precipitates a broad range of cognitive deficits. Despite considerable recent progress, the specific synaptic function of neuroligin-1 (NL1) remains unclear. A current model proposes that NL1 acts exclusively to mature pre-existent synaptic connections in an activity-dependent manner. A second element of this activity-dependent maturation model is that an alternate molecule acts upstream of NL1 to initiate synaptic connections. SynCAM1 (SC1) is hypothesized to function in this capacity, though several uncertainties remain regarding SC1 function. Using overexpression and chronic pharmacological blockade of synaptic activity, we now demonstrate that NL1 is capable of robustly recruiting synapsin-positive terminals independent of synaptic maturation and activity in 2-week old primary hippocampal neuronal cultures. We further report that neither SC1 overexpression nor knockdown of endogenous SC1 impacts synapsin punctum densities, suggesting that SC1 is not a limiting factor of synapse initiation in maturing hippocampal neurons *in vitro*. Consistent with these findings, we observed profoundly greater recruitment of synapsin-positive presynaptic terminals by NL1 than SC1 in a mixed-culture assay of artificial synaptogenesis between primary neurons and heterologous cells. Collectively, our results contend multiple aspects of the proposed model of NL1 and SC1 function and motivate an alternative model whereby SC1 may mature synaptic connections forged by NL1. Supporting this model, we present evidence that combined NL1 and SC1 overexpression triggers excitotoxic neurodegeneration through SC1 signaling at synaptic connections initiated by NL1.

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Keywords

Neurologin; Neurexin; SynCAM; Synaptogenesis; Synapse initiation; Activity-dependent

1. Introduction

Adhesion molecules orchestrate the accurate alignment of pre- and postsynaptic specializations to enable spatiotemporally precise neurotransmission (Dalva et al., 2007). Postsynaptic neuroligins (NLs), and their cognate presynaptic receptors neurexins (NRXs), are two such families of synapse-specific adhesion molecules critically involved in establishing central nervous system connectivity (Dean and Dresbach, 2006). Disruption of NL-NRX signaling precipitates a broad range of cognitive deficits, including motor, learning, and social impairments (Jamain et al., 2008; Kim et al., 2008; Hines et al., 2008; Blundell et al., 2009; Blundell et al., 2010; Dahlhaus et al., 2010). Moreover, specific NL and NRX mutations have been repeatedly implicated in autism spectrum disorders (Südhof, 2008). Understanding these cognitive alterations will ultimately require defining the specific roles of NL-NRX signaling.

Considerable evidence links NL-NRX signaling to synapse initiation. In an assay of artificial synaptogenesis, neuroligin-1 (NL1) expressed in nonneuronal cells is sufficient to induce presynaptic differentiation in contacting axons of cocultured neurons (Scheiffele et al., 2000; Fu et al., 2003). Moreover, NL1 overexpression in primary neuronal cultures potently increases synapse densities and can increase spontaneous miniature postsynaptic current (mini) frequencies, while NL1 knockdown inhibits normal synaptogenesis (Dean et al., 2003; Prange et al., 2004; Chih et al., 2005; Shipman et al., 2011). Critically, NL1 accumulates within seconds at sites of nascent axodendritic contact, temporally consistent with synapse initiation (Barrow et al., 2009).

In vivo knockout results conflict with the sum of *in vitro* data, however. NL1 deletion strongly impairs neurotransmission while minimally impacting synapse densities, suggesting that NL1 may primarily contribute to synapse function and not formation (Varoqueaux et al., 2006; Chubykin et al., 2007; Blundell et al., 2010). A prevailing model reconciling these datasets posits that NL1 acts exclusively to mature and stabilize pre-existent connections in a *N*-methyl-D-aspartate receptor (NMDAR)-mediated activity-dependent manner, and so does not directly drive synapse initiation (Chubykin et al., 2007). While consistent with several knockout and *in vitro* findings, the activity-dependent maturation model cannot account for the striking results from the artificial synaptogenesis assay, which is devoid of postsynaptic activity. Further, evidence directly supporting this model, in which chronic NMDAR blockade ablated NL1-mediated gains in synapsin punctum densities, was confounded by nonspecific effects of NMDAR blockade (Chubykin et al., 2007).

The activity-dependent maturation model further requires that an alternate molecule acts upstream of NL1 to initiate synaptogenesis. Adhesion molecule SynCAM1 (SC1) is hypothesized to function in this capacity (Chubykin et al., 2007; Robbins et al., 2010). Supporting this hypothesis, SC1 exhibits punctate somatodendritic expression (Fogel et al., 2011), contributes to axonal growth cone dynamics (Stagi et al., 2010; Fogel et al., 2011), can rapidly assemble at sites of nascent axodendritic contact (Stagi et al., 2010; Fogel et al., 2011), and can increase mini frequency (Sara et al., 2005; Fogel et al., 2007) in immature primary hippocampal neurons. Moreover, SC1 is active in the artificial synaptogenesis assay (Biederer et al., 2002; Sara et al., 2005; Fogel et al., 2007; Biederer and Scheiffele, 2007; Hoy et al., 2009; Fogel et al., 2011), and SC1 deletion *in vivo* yields small but significant reductions in synapse densities (Robbins et al., 2010). Despite this substantial evidence for a

role in early phases of synaptogenesis, however, SC1 overexpression fails to increase synapse densities in both immature and maturing primary hippocampal cultures (Sara et al., 2005; Fogel et al., 2007; Chubykin et al., 2007). Likewise, an eightfold increase in SC1 expression in transgenic mice yields only a modest ~25% increase in synapse density (Robbins et al., 2010) compared to the ~100% increase observed with only a twofold increase in NL1 expression (Dahlhaus et al., 2010). The ability of SC1 to drive synapse initiation, as well as the endogenous role of SC1 relative to NL1 in developmental synaptogenesis, thus remains unclear.

Here we have examined multiple aspects of the activity-dependent maturation model of NL1 function. In contrast to this model, we demonstrate that NL1 can robustly increase the density of synapsin-positive connections independent of synaptic maturation and NMDAR-mediated activity in 2-week old primary hippocampal neuronal cultures. Data reconciling overexpression and artificial synaptogenesis experiments further confirms a greater impact of NL1 than SC1 on terminal recruitment *in vitro*. These results, together with those from combined NL1/SC1 perturbations, support an alternative model whereby SC1 may mature synaptic connections forged by NL1.

2. Experimental procedures

2.1 Antibodies and DNA constructs

The following primary antibodies were used: chicken anti-influenza hemagglutinin epitope (HA; Millipore AB3254; 1:250; Jennings et al., 2006), rabbit anti-synapsin I (Millipore AB1543; 1:500; Scheiffele et al., 2000; Washbourne et al., 2002; Biederer and Scheiffele, 2007), mouse anti-MAP2 (Sigma Aldrich M9942; 1:200; Kaech and Banker, 2006; Biederer and Scheiffele, 2007), rabbit anti-FLAG (Affinity Bioreagents PA1-984B; 1:500; Onoguchi et al., 2010), and sheep anti-actin (Millipore AB3265; 1:1000; Lionnet et al., 2011). Secondary antibodies used in immunocytochemistry were Alexa Fluor 647:goat anti-mouse IgG (Invitrogen A-21235; 1:1000), Alexa Fluor 555:goat anti-chicken IgG (Invitrogen A-21437; 1:1000), Alexa Fluor 488:goat anti-rabbit IgG (Invitrogen A-11008; 1:1000), and Alexa Fluor 555:goat anti-rabbit IgG (Invitrogen A-21428; 1:1000). Secondary antibodies used in immunoblotting were horseradish peroxidase (HRP) conjugates and included: HRP:goat anti-rabbit IgG (Invitrogen SJ29096, 1:10000) and HRP:rabbit anti-sheep IgG (Millipore 12-342; 1:1000).

For all NL1 experiments, the murine NL1(AB) isoform was used. NL1(AB) and NLswap constructs were kindly provided by Dr. Peter Scheiffele and previously described (Scheiffele et al., 2000; Chih et al., 2004). Briefly, the HA epitope tag was inserted downstream of the N-terminal signal sequence in the extracellular domain of NL1 or NLswap, creating HA:NL1 and HA:NLswap, respectively. HA:NL1 and HA:NLswap were expressed under control of the chicken beta actin promoter of the pCAGGS vector. The lentiviral vectors LentiLox3.7 (LL3.7) and LL3.74 were kindly provided by Dr. Carlos Lois and used for expression of soluble EGFP or DsRed2, respectively, as well as in RNA-interference (RNAi) experiments to express short hairpin RNA sequences under control of the U6 promoter. The murine SC1 expression construct was kindly provided by Dr. Thomas Biederer and previously described (Fogel et al., 2007). Briefly, the FLAG epitope tag was inserted within the extracellular domain of SC1, and the resulting FLAG:SC1 was expressed using the pCAGGS vector. The SCΔIg expression construct was kindly provided by Dr. Thomas Südhof and was previously described (Biederer et al., 2002). Briefly, the three Ig domains of SC1 were deleted, and the resulting SCΔIg was expressed under control of the CMV promoter of the pCMV5 vector.

For SC1 knockdown, the short hairpin “shSC1” targeting the sequence CAGAAGGAGGACAGAACAA within the murine SC1 coding sequence was designed in accordance with published criteria (Reynolds et al., 2004). The efficacy of shSC1 was tested by coexpression of FLAG:SC1 and shSC1 (at a ratio of 1:3) in HEK293FT cells and immunoblotting against the FLAG epitope. For RNAi control experiments, a scrambled version of a separate short hairpin sequence was used.

2.2 Cell culture and transfection

All experiments were in accordance with the guidelines established by the University of Pittsburgh's Institutional Animal Care and Use Committee. For experiments in primary neuronal cultures, hippocampi were dissociated from E18-19 C57BL/6 mouse embryos and plated at 30,000 cells/cm² on confluent mouse neocortical glial monolayers previously prepared on glass coverslips (Carolina Biological Supply) coated with poly-D-lysine. Cultures were maintained in neurobasal medium supplemented with 2% B27 (Invitrogen), 1% penicillin-streptomycin, and 0.5 mM L-glutamine and kept at 37°C in a 95% O₂/5% CO₂ incubator. One quarter of the total culture medium was exchanged for fresh medium every 2–3 days. Cultures were maintained in 3 μM cytosine arabinofuranoside beginning at day *in vitro* (DIV) 2 to prevent glial overgrowth. Under these conditions, neuronal cultures regularly established extensive axodendritic networks over the confluent glial monolayer within a few days of dissociation, with limited or no fasciculation or processes, clumping of somas, or segmentation of neuronal processes up to at least 3 weeks in culture. Neurodegeneration was identified based on a combination of phenotypic changes observed under visual examination, including fragmentation and swelling of neurites, stunted neurite growth, and minimal overall arborization (Mattson et al., 1988).

Neurons were transfected at DIV9-10 using Lipofectamine 2000 (Invitrogen) essentially as described by the manufacturer with the following modifications: for each 35 mm dish, 2 μg total DNA was mixed with 1.5 μl Lipofectamine 2000 reagent in 80 μl total volume of Opti-MEM (Invitrogen). Transfections were carried out for 1 hr, followed by a 5 min wash. Except where noted in Results, transfected cells exhibited normal morphologies with numerous, gradually tapering primary dendrites, extensive axodendritic arborization, and no swelling or segmentation of processes. For NMDAR-mediated activity blockade, 100 μM D-AP5 was added to cultures at the time of transfection and renewed every 1–2 days. Similar to Chubykin et al. (2007), we observed no attenuation of transgene expression with NMDAR blockade.

HEK293FT cells (Invitrogen) were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. For mixed-culture assays of artificial synaptogenesis, hippocampi were dissociated as above and plated directly onto glass coverslips coated with poly-D-lysine and laminin. Cultures were maintained in a manner identical to neuron/glia cultures described above except initial plating medium contained 25 μM glutamate. HEK293FT cells were transfected at neuronal DIV8-9 and seeded 1–2 days later at 250 cells/cm² onto pure neuronal cultures.

2.3 Immunoblotting

Protein was extracted from HEK293FT cell cultures using ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and complete protease inhibitor cocktail (Roche)). Protein concentrations were quantified using a modified Lowry assay (Bio-Rad Laboratories). Cell lysates were prepared in Laemmli buffer, denatured at 95°C for 3 min, and equal total masses loaded into 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes using a wet transfer

method. Membranes were blocked in 5% instant nonfat dry milk and incubated with primary and secondary antibodies prepared in 2% milk solutions. All incubations were carried out for 1–3 hr at room temperature or overnight at 4°C. Protein signals were detected using HRP-mediated chemiluminescence.

2.4 Immunocytochemistry

Cells were fixed in a 4% paraformaldehyde/4% sucrose, phosphate-buffered saline (PBS) solution at 37°C for 10 min. Fixed cells were permeabilized with a 0.3% Triton X-100 PBS solution. Nonspecific binding was blocked using a 1–3 hr room temperature or overnight 4°C incubation in a 5% BSA PBS solution. Cells were sequentially incubated with primary and secondary antibodies diluted in a 1% BSA PBS solution for 1–3 hr each at room temperature. For the triple immunostaining of MAP2, HA, and synapsin performed for Figure 1, cells were fixed in ice-cold methanol.

2.5 Imaging and analysis

Images were collected on upright Olympus Fluoview 1000 and 500 confocal microscopes using a 60X oil-immersion objective and 1X digital zoom in maximum intensity projections of 0.5 μm -step z-stacks at 2048 $\times$ 2048 resolution, unless otherwise specified. Equivalent settings were used across all images for each experiment. For all of the series of immunostained images presented in figures, the rightmost images show a merge of the individual channels displayed to the left. Boxed regions of interest defined in merged images are enlarged in insets.

Synapsin punctum densities were quantified in NIH ImageJ using the analyze particles function and a manual per neurite background subtraction procedure (Glynn and McAllister, 2006). Only puncta completely or partially overlapping with thresholded dendritic soluble EGFP or MAP2 signals were counted in densities. Mean intensities of immunostained HA:NL1 within a dendritic branch were quantified after per neurite background subtraction and measured as the total mean intensity of all pixels within a defined dendritic branch segment. Dendrites were defined either by positive MAP2 immunoreactivity (Cáceres et al., 1984) or morphologically, based upon thick diameters and clear presence of spiny protrusions. Dendritic spines were identified and counted manually, with protrusions longer than $\sim 5 \mu\text{m}$ or displaying clear branching excluded from counts.

For mixed-culture assays of artificial synaptogenesis, the percentage of HEK293FT cell area colocalized with suprathreshold synapsin immunofluorescence was quantified as a metric for induction of presynaptic differentiation (Biederer and Scheiffele, 2007). Briefly, synapsin fluorescence was thresholded by identifying 10 background points and subtracting their mean grayscale intensity +2 standard deviations from the measured grayscale intensity of each pixel comprising the synapsin image. Fluorescence of HEK293FT cell cotransfection marker EGFP or DsRed2 was then thresholded to define the HEK293FT cell outline and used to confine the region of suprathreshold synapsin immunofluorescence quantification.

2.6 Electrophysiology

Whole-cell patch-clamp recordings of DIV12–14 hippocampal neurons were made at room temperature using an Axopatch 200A amplifier. The intracellular solution contained (in mM) 135 CsCl, 10 HEPES, 1 EGTA, and 4 Mg-ATP (pH 7.4) and the extracellular solution contained (in mM) 150 NaCl, 4 KCl, 2 MgCl_2 , 10 glucose, 10 HEPES, and 2 CaCl_2 , yielding a junction potential of $\sim 5.7 \text{ mV}$ for which correction was made prior to recording. Compensation for series resistance was not applied. Neurons were voltage-clamped at -70 mV and minis were recorded in the presence of $1 \mu\text{M}$ TTX. Data were low-pass filtered at 2

kHz and sampled at 20 kHz. Minis were identified using Clampfit 9 (Molecular Devices) with an amplitude threshold ~4 times greater than the RMS noise level and confirmed visually. A 1 kHz posthoc filter was applied to representative traces presented in figures for greater visual clarity.

2.7 Statistical analysis

For imaging experiments, statistical comparisons among groups were made using two-tailed t-tests for 2 groups and one-way ANOVAs for 3 groups, with posthoc Tukey-test completed upon significant ANOVA results ($p < 0.05$). Statistical significance is reported as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. In electrophysiology experiments, statistical comparisons of mini frequencies among groups were made using a one-way ANOVA for 3 groups, as above. Statistical comparisons of mini amplitudes among groups were made by randomly sampling 100 minis from each condition and performing two-sample Kolmogorov-Smirnov tests with Bonferroni correction for multiple comparisons. Column numbers in each figure indicate the number of cells quantified per condition, with error bars denoting standard error of the mean (SEM). For each experiment, 2–4 cultures were used per condition or experimental group. All statistical comparisons were performed using OriginPro 8.0 and Matlab. Figures were prepared using NIH ImageJ, OriginPro 8.0, and Adobe Illustrator CS2.

3. Results

3.1 Characterization of NL1 overexpression

In prior accounts of NL1 overexpression, the resulting increase in synapsin punctum densities has varied considerably, from a modest twofold increase in synapsin punctum density with NL1 overexpression (Boucard et al., 2005) to a pronounced fivefold increase (Dean et al., 2003). To what extent this variation in terminal recruitment is determined by the actual level of NL1 expression *in vitro* has not been reported. We thus examined three distinct levels of NL1 overexpression to explore directly the dependence of terminal recruitment on NL1 expression. Cultures were transfected at DIV9–10 using the Lipofectamine 2000 reagent and 500 ng of the NL1 expression vector for “high overexpression”, 100–200 ng for “overexpression”, or 20–50 ng for “low overexpression” conditions. Resulting densities of recruited presynaptic terminals along dendrites of transfected neurons were assessed 3–4 days later by immunostaining for synapsin (Fig. 1A–C), a well-accepted marker of synaptic connections. We additionally analyzed synapsin punctum densities along dendrites of untransfected cells neighboring neurons overexpressing NL1 to quantify basal terminal recruitment. Importantly, the chosen experimental timeframe corresponds to a period of intense synaptogenesis and parallels numerous prior studies (Dean et al., 2003; Prange et al., 2004; Chih et al., 2004; Chih et al., 2005; Sara et al., 2005; Boucard et al., 2005; Chubykin et al., 2007). To ensure comparable transfection efficiencies across each overexpression level, the total mass of DNA per transfection was kept constant by adjusting the mass of a cotransfected vector lacking coding sequences.

As expected, the average intensity of exogenous NL1 increased with transfection of greater masses of the NL1 expression vector (Fig. 1D), verifying distinct levels of NL1 overexpression. With higher overexpression levels, exogenous NL1 expanded across greater regions of dendritic membrane but still exhibited clear clustering at distinct points along dendritic shafts and in spine heads (Fig. 1A). Increasing the level of NL1 overexpression produced a clear and highly significant increase in average synapsin punctum density (Fig. 1D), with a significant logarithmic relationship observed between exogenous NL1 expression and synapsin puncta recruitment (Fig. 1E). This dose-dependent relationship compellingly argues that NL1 is specifically involved in increasing the density of synaptic

connections. Moreover, the large range of synapsin punctum densities induced by different levels of NL1 overexpression may account for the large disparity in synapse gains previously observed. This experiment additionally established overexpression parameters for further testing.

3.2 Functional evaluation of NL1-mediated terminal recruitment

In interpreting the results from Figure 1, it is not possible to determine whether NL1 is capable of driving synapse initiation, maturation (and stabilization), or both. Similarly, it cannot be determined from the data in Figure 1 whether the recruitment of synapsin-positive terminals is primarily a direct result of transsynaptic NL1 signaling or an indirect result of intracellular clustering of other synapse-initiating components, or both. To address these uncertainties, we next examined synapsin punctum densities and synaptic function among three conditions: high NL1 overexpression, NLswap expression, and control EGFP expression (Fig. 2). NLswap is a NL1 mutant incapable of transsynaptic signaling (Scheiffele et al., 2000; Dean et al., 2003) but able to cluster postsynaptic components (Chih et al., 2005), and should thus help dissociate transsynaptic from intracellular NL1 signaling. To assess synapse functionality, we employed whole-cell patch-clamp electrophysiology to record minis. Using this technique, an increase in mini frequency with high NL1 overexpression would most likely reflect an increase in the number of functional synapses, suggesting that NL1 increases synaptic connection densities via mechanisms that include a maturation and stabilization pathway. Alternatively, no change in frequency would instead reveal that NL1 is capable of forging immature synaptic connections lacking the appropriate neurotransmitter receptors postsynaptically or neurotransmitter release machinery presynaptically, or both.

As observed above (Fig. 1A), high NL1 overexpression induced pronounced recruitment of synapsin-positive terminals, with synapsin puncta clearly outlining all dendritic processes (Fig. 2A). Neither NLswap-expressing neurons (Fig. 2E) nor control neurons expressing EGFP alone (Fig. 2C) displayed this level of synapsin recruitment. Quantification confirmed that high NL1 overexpression induced a highly significant, roughly fourfold increase in synapsin punctum densities compared to both EGFP control and NLswap conditions (Fig. 2G). High NL1 overexpression thus recruits synapsin-positive terminals primarily through transsynaptic signaling. High NL1 overexpression also potently increased dendritic spine densities compared to EGFP control and NLswap conditions (Fig. 2H).

Strikingly, the robust gains in synapsin punctum densities observed with high NL1 overexpression were not coupled with an increase in mini frequencies (Fig. 2B). Rather, NL1-overexpressing neurons displayed frequencies similar to NLswap-expressing neurons, which were roughly fivefold to sixfold lower than in control cells (Fig. 2J). This disparity between structural and functional effects clearly evinces NL1's capacity to recruit new synaptic connections independent of synaptic maturation. Moreover, the strong trend towards decreased mini frequencies suggests that NL1 overexpression may disrupt normal α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) recruitment, as shown previously using immunostaining (Chih et al., 2005). Supporting this conclusion, high NL1 overexpression also significantly reduced mini amplitudes from control levels (Fig. 2J). Our results are thus inconsistent with an exclusive function of NL1 in synapse maturation and instead reveal a robust ability of NL1 to forge immature synaptic connections independent of postsynaptic maturation.

3.3 Activity dependence of NL1-mediated synapse gains

While the synaptic connections formed by high NL1 overexpression are functionally immature, they may still harbor significant NMDAR-mediated activity in line with the

activity-dependent maturation model of NL1. Thus, to evaluate directly the NMDAR-mediated activity-dependence of NL1 function, we examined the effect of chronic NMDAR blockade on NL1-mediated synaptic gains (Fig. 3). To achieve chronic blockade, we added 100 μ M D-AP5 to the culture medium every 1–2 days from the time of transfection, as previously performed (Chubykin et al., 2007). Contrary to previous findings, however, we observed no significant effect of chronic NMDAR blockade on synapsin punctum densities (Fig. 3C). Instead, dendrites of NL1-overexpressing neurons in both conditions exhibited extensive recruitment of synapsin-positive presynaptic terminals (Fig. 3A,B), as above (Fig. 1A,2A). Chronic NMDAR blockade did limit the effect of high NL1 overexpression on dendritic spine densities, however (Fig. 3D). Our results thus partially dissociate activity-independent initiation of immature synaptic connections by NL1 from NMDAR-mediated activity-dependent spine morphogenesis.

3.4 Overexpression and knockdown of SC1

While our findings thus far strongly suggest that NL1 is capable of initiating new synaptic connections, they do not rule out potential contributions of SC1 prior to NL1 function. Currently, a comprehensive acute gain- and loss-of-function evaluation of SC1 *in vitro* is lacking, and may help clarify the contribution of SC1 to basal synaptogenesis. We thus designed a short-hairpin sequence targeting murine SC1 for RNAi knockdown. Due to a lack of sufficiently selective SC1 antibodies available, we evaluated the efficacy of our designed hairpin through coexpression with FLAG:SC1 in HEK293FT cells. Coexpression of the empty RNAi vector and a control hairpin not targeting SC1 were included as negative controls. Using this approach, we consistently observed pronounced knockdown of SC1 expression by the designed short-hairpin compared to control hairpin and empty vector controls (Fig. 4), thus confirming viability of this strategy to acutely perturb SC1 expression.

Consistent with prior reports (Sara et al., 2005; Chubykin et al., 2007), SC1 overexpression did not alter synapsin punctum or dendritic spine densities from basal levels (Fig. 5A,B). In particular, we note that none of the SC1-overexpressing neurons exhibited the remarkable synapsin puncta outlining that we observed with NL1 overexpression (for example, compare Fig. 2A,G with Fig. 5A,D). Further, acute knockdown of endogenous SC1 expression over 3–4 days revealed that normal levels of SC1 expression are not essential for basal terminal recruitment or spine morphogenesis in primary hippocampal neuronal cultures (Fig. 5C). Quantification of synapsin punctum and spine densities indeed confirmed no significant difference among SC1 overexpression, EGFP control, and SC1 knockdown conditions (Fig. 5D,E). Our data thus do not support the hypothesis that SC1 is a limiting factor of synaptogenesis acting upstream of NL1 *in vitro*.

3.5 Recruitment of presynaptic terminals by NL1 versus SC1 in a mixed-culture assay of artificial synaptogenesis

The inability of SC1 to initiate new synaptic connections in primary neuronal cultures is at odds with previous reports of the ostensibly similar ability of NL1 and SC1 to recruit presynaptic terminals in the artificial synaptogenesis assay. To date, however, the only direct comparison between NL1 and SC1 in this artificial synaptogenesis assay focused exclusively on the functionality of recruited terminals (Sara et al., 2005). We thus performed a complementary morphological analysis of terminal recruitment by NL1 versus SC1 to help resolve the apparent discrepancy between overexpression and artificial synaptogenesis assay results.

To compare the ability of NL1 and SC1 to recruit presynaptic terminals, HEK293FT cells were transfected to express either HA:NL1 or FLAG:SC1 (with cotransfection marker DsRed2) and cocultured with primary hippocampal neurons for 1–2 days before

immunostaining for synapsin. Neurons were cultured at lower densities than previously employed (Sara et al., 2005; Hoy et al., 2009) to minimize the colocalization of HEK293FT cells with interneuronal synapses, as well as to preclude saturation of the artificial synaptogenesis phenomenon. Under these conditions, NL1-expressing HEK293FT cells recruited far greater amounts of synapsin than did SC1-expressing cells (Fig. 6). Quantification of the fractional HEK293FT cell area colocalized with suprathreshold synapsin immunofluorescence indeed revealed NL1-expressing cells to recruit an order of magnitude more synapsin than SC1-expressing cells under these experimental conditions (Fig. 6C). We consistently observed high heterologous expression of both NL1 and SC1 using both surface-specific immunocytochemistry (data not shown) and whole-cell western blotting (Fig. 4 and data not shown). Our results thus uncover a substantial difference between NL1 and SC1 potencies in the mixed-culture assay of artificial synaptogenesis when using low neuronal culture densities, in direct accordance with our overexpression and knockdown results.

3.6 Combined overexpression of NL1 and SC1

Our results thus far demonstrate a strong capacity of NL1, and not SC1, to initiate new synaptic connections in 2-week old primary hippocampal neuronal cultures. The enhanced mini frequencies previously observed with SC1 overexpression in immature neurons (Sara et al., 2005; Fogel et al., 2007) thus likely reflect a role in receptor recruitment (Hoy et al., 2009). These distinct roles of NL1 and SC1 may potentially complement one another at single synapses, leading to SC1-mediated maturation of NL1-initiated synaptic connections, as previously proposed (Sara et al., 2005). Surprisingly, this attractive model of coordinated signaling between synaptic adhesion molecules remains untested.

To begin examining potential cooperation of NL1 and SC1 in synapse formation and function, we compared effects of NL1 overexpression versus NL1/SC1 co-overexpression in two-week old primary hippocampal neuronal cultures. Both the total mass of DNA and the individual masses of expression vectors used in these transfections were kept constant. Strikingly, co-overexpression of NL1 and SC1 triggered marked neurodegeneration in 11 of 11 neurons examined 3–4 days post transfection (8 representative neurons shown in Fig. 7B). In comparison, 12 of 12 neurons examined overexpressing NL1 alone displayed expansive arborization and no detectable segmentation or swelling of dendrites or axons (8 representative neurons shown in Fig. 7A). Moreover, neurons overexpressing SC1 alone in earlier experiments (Fig. 5A) also did not exhibit these clear signs of neurodegeneration. These results thus collectively suggest that the neurodegeneration observed is a specific effect of coordinated signaling of NL1 with SC1.

All aspects of the neurodegenerative pattern observed in neurons co-overexpressing NL1 and SC1 phenocopy classical signs of glutamatergic excitotoxic degeneration in primary pyramidal neurons (Mattson et al., 1988). Thus, to directly test the dependence of NL1/SC1-mediated neurodegeneration on glutamatergic activity, we repeated the co-overexpression experiment and bath-applied 100 μ M D-AP5 from the time of transfection, as above (Fig. 3). Remarkably, chronic NMDAR blockade precluded all signs of neurodegeneration (Fig. 8B), suggesting that NL1/SC1 coordination does indeed trigger NMDAR-mediated activity-dependent excitotoxicity. Neither synapse densities nor dendritic spine morphogenesis were significantly different between NL1-overexpressing and NL1/SC1-co-overexpressing neurons in AP5 (Fig. 8C,D), consistent with SC1 involvement in synapse function and not formation. We also note that, again, chronic NMDAR blockade did not prevent the dramatic recruitment of synapsin by NL1 but did limit spine morphogenesis, consistent with the above results (Fig. 3).

We thus present the first evidence for SC1-mediated maturation of NL1-initiated synapses. What we cannot determine from these results, however, is whether this cooperative effect requires transsynaptic signaling by SC1. To address this uncertainty, we co-overexpressed NL1 with SC Δ Ig, a SC1 mutant incapable of transsynaptic signaling (Biederer et al., 2002; Sara et al., 2005). Under normal culture conditions (no NMDAR blockade), we observed no visible signs of neurodegeneration in 7 of 7 neurons overexpressing NL1 alone (Fig. 9A) or in 11 of 11 neurons overexpressing NL1 with SC Δ Ig (Fig. 9B). Moreover, neither synapse densities nor dendritic spine morphogenesis significantly differed between neurons overexpressing NL1 alone or with SC Δ Ig (Fig. 9C,D), further supporting the capacity of NL1 to drive synapse formation independent of SC1 signaling. Our results thus intriguingly suggest that transsynaptic SC1 signaling is required to trigger NMDAR-mediated activity-dependent neurodegeneration in neurons co-overexpressing NL1 with SC1.

4. Discussion

In this study, we examined the abilities of synaptic adhesion molecules NL1 and SC1 to recruit synapsin-positive terminals in primary hippocampal neuronal cultures. Critically, we found that NL1 overexpression robustly triggered terminal recruitment independent of synapse maturation and activity, directly challenging the model that NL1 functions exclusively to mature pre-existent synapses in an activity-dependent manner (Chubykin et al., 2007). In contrast, neither overexpression nor knockdown of SC1 altered synapsin punctum or spine densities, while direct comparison of NL1 and SC1 in the artificial synaptogenesis assay revealed minimal synaptogenic potential of SC1-expressing cells. Co-overexpression of SC1 with NL1 likewise did not enhance NL1-mediated terminal recruitment but did precipitate NMDAR-mediated activity-dependent neurodegeneration, congruent with an alternative model whereby SC1 matures new synaptic connections forged by NL1.

4.1 Interpretation of NL1 overexpression

Comparison of experimental methods and corresponding results across studies is crucial to accurate interpretation of gain- and loss-of-function perturbations. Most studies of acute NL1 overexpression have not quantified the amount of exogenous NL1 expressed, however, curtailing direct comparison of overexpression levels and results. We now demonstrate a strong ($R^2=0.75$) and highly significant ($p<0.001$) correlation between synapsin punctum densities and NL1 overexpression level in dissociated hippocampal neurons. This finding enables indirect comparison of the NL1 overexpression levels used throughout our study to overexpression levels previously reported. Specifically, the highest level of NL1 overexpression ("high overexpression") in our study triggered an approximate fourfold increase in presynaptic terminal recruitment, which is well within the range observed by other investigators using similar culture techniques (see, for example: Dean et al., 2003; Chih et al., 2004; Chih et al., 2005). Thus, our current results complement these previous studies. In particular, Chih et al. (2005) observed increased synaptic recruitment of PSD-95 and NMDARs but dispersion of AMPARs with NL1 overexpression, providing a structural correlate for the functional reduction in spontaneous synaptic activity that we observed. The convergence of our results with those of Chih et al. (2005), as well as the significant effect of chronic NMDAR blockade on NL1-mediated spine morphogenesis that we observed, suggests that the NL1 overexpression protocol that we employed did not broadly disrupt postsynaptic differentiation, as observed with high NL2 overexpression (Graf et al., 2004). Rather, NL1 overexpression led to specific deficits in AMPAR recruitment and synapse maturation, reflecting independent NMDAR and AMPAR recruitment pathways (Barrow et al., 2009). Südhof and colleagues have similarly noted a differential effect of NL1 perturbation on NMDAR- versus AMPAR-mediated currents (Chubykin et al., 2007).

4.2 Contribution of NL1 to activity-independent synapse initiation

Numerous lines of evidence support our principal finding that NL1 can specifically drive synapse formation independent of synaptic maturation and activity. First, the striking gains in synapse and spine densities triggered by high NL1 overexpression were not coupled with an increase in mini frequency. The simplest interpretation of this disparity is that the new synaptic connections were functionally immature, which is incongruous with exclusive activity-dependent NL1-mediated synapse maturation. Second, high NL1 overexpression significantly reduced mini amplitudes. This, together with a strong trend towards decreased frequencies, strongly suggests that overexpressed NL1 failed to mature synaptic connections, again incongruous with exclusive activity-dependent NL1-mediated synapse maturation. Third, chronic NMDAR blockade had no effect on NL1-mediated synapse formation despite specifically reducing NL1-mediated spine morphogenesis. Not only do these data support an NMDAR activity-independent synaptogenic capacity of NL1, but they further suggest that the spine morphogenesis associated with synapse maturation can be dissociated from synapse initiation. Fourth, artificial clustering of endogenous or exogenous NRX by recombinant NL1 to simulate NL1/NRX binding in the absence of synaptic activity can trigger presynaptic differentiation (Dean et al., 2003). Fifth, NL1 is recruited within seconds to nascent axodendritic contact sites (Barrow et al., 2009), preceding recruitment of PSD-95, NMDARs, and AMPARs (Friedman et al., 2000; Bresler et al., 2001; Washbourne et al., 2002; Gerrow et al., 2006; Barrow et al., 2009). Lastly, NL1 is able to recruit active zone scaffolding and maintain dendritic filopodia – processes associated with initiation of nascent synaptic connections – independent of NMDAR activity (Wittenmayer et al., 2009; Chen et al., 2010).

4.3 Contribution of NL1 to multiple synaptic processes

Our data thus add to a substantial body of findings evincing the robust ability of NL1 to drive activity-independent synapse initiation in neurons. It is critical to note, however, that this ability and our current study do not exclude additional, activity-dependent capacities in synapse maturation, stabilization, and/or plasticity. Indeed, NMDAR-mediated activity is necessary for NL1-mediated maturation of presynaptic terminals (Wittenmayer et al., 2009) and structural stabilization of dendritic filopodia (Chen et al., 2010). Moreover, both chronic and acute *in vivo* perturbation of NL1 is linked to modulation of NMDAR-mediated activity and NMDAR-mediated activity-dependent plasticity (Chubykin et al., 2007; Kim et al., 2008; Blundell et al., 2010; Dahlhaus et al., 2010). NL1 is thus most likely an integral driver and modulator of numerous activity-independent and activity-dependent synaptic processes.

What evidence argues against the ability of NL1 to drive activity-independent synapse initiation? Foremost, Südhof and colleagues reported that chronic NMDAR blockade precluded NL1-mediated synapse gains (Chubykin et al., 2007). In their study, however, chronic NMDAR blockade also significantly reduced basal synapse densities in control neurons, confounding a direct link between NL1 overexpression and NMDAR-mediated activity. Further, NL1 overexpression still triggered a significant, though less pronounced, increase in synapse densities under chronic NMDAR blockade (Chubykin et al., 2007, and recently replicated by Ko et al., 2011). In contrast, our results clearly evince the ability of NL1 to increase synapse densities independent of NMDAR-mediated activity. Moreover, the morphological data that we present are complemented by recent work from Nicoll and colleagues in which NL1 overexpression in slice culture enhanced evoked currents independent of synaptic activity (Shipman et al., 2011). The collection of these results is thus concordant with involvement of NL1 in multiple synaptic functions.

Lack of a pronounced loss of synapse densities in NL knockout mice (Varoqueaux et al., 2006) may also suggest that NLs are not involved in synapse initiation. Such direct

interpretation of knockout results can be problematic, however, owing to complex compensatory changes. For example, NL1-NL3 knockout significantly decreases protein levels of the potassium-chloride cotransporter KCC2, which contributes to the crucial developmental switch in chloride reversal potential (Varoqueaux et al., 2006). Because NL knockout alters synaptic activity, confounding homeostatic shifts are also expected. For example, Gibson et al. (2009) reported that, while inhibitory transmission is impaired in NL2 knockout mice, reciprocal excitatory transmission is augmented. Redundancy in adhesion complexes at excitatory synapses, including NLs-NRXs, SCs, leucine-rich repeat transmembrane proteins (LRRTMs)-NRXs, EphB2s-EphrinBs, and netrin-G-ligands (Dalva et al., 2007; Linhoff et al., 2009; de Wit et al., 2009) must also be considered. Indeed, NL1, NL3, LRRTM1, and LRRTM2 may compensate for one another in knockout animals (Ko et al., 2011; Soler-Llavina et al., 2011). Remarkably, the only known adhesion complex regulating inhibitory γ -aminobutyric acid (GABA)-ergic synapses throughout the brain is NL2-NRX (Varoqueaux et al., 2004; Graf et al., 2004; Levinson et al., 2005; Patrizi et al., 2008; Pouloupoulos et al., 2009). This apparent lack of adhesion complex redundancy at GABAergic synapses may explain why *in vivo* perturbations of NL2 (Hines et al., 2008; Pouloupoulos et al., 2009) precipitate greater changes than perturbation of either NL1 or NL3 (Chubykin et al., 2007; Tabuchi et al., 2007; Blundell et al., 2010). Indeed, NL1-NL3 knockout most prominently disrupts inhibitory synapse formation and function (Varoqueaux et al., 2006), similar to NL2 knockout alone (Pouloupoulos et al., 2009). The sum of *in vivo* results thus does not conclusively exclude involvement of NLs in synapse initiation.

4.4 Synaptic coordination of NL1 and SC1

Defining the specific contributions of NL1 to synaptic and neural development will require investigating coordinated signaling between NL1 and alternate adhesion molecules. Toward this end, our results challenge the proposed action of SC1 in synapse initiation upstream of NL1. In particular, our results from acute gain- and loss-of-function analyses of SC1 complement other studies to show that SC1 is not a limiting factor of synapse formation *in vitro* (Sara et al., 2005; Fogel et al., 2007; Chubykin et al., 2007). These results are further supported by recent demonstration that extracellular SC1 interactions are necessary to maintain synapse size, and not synaptic densities, in primary hippocampal neurons (Fogel et al., 2011). Additionally, we now reveal a pronounced difference between NL1 and SC1 in the mixed-culture assay of artificial synaptogenesis, demonstrating minimal synaptogenic potential of SC1. This observation reaffirms qualitative comparison of several prior investigations in which NL1-expressing cells exhibit far greater recruitment of presynaptic markers than SC1-expressing cells (compare, for example: Biederer et al., 2002; Sara et al., 2005; Chubykin et al., 2005; Fogel et al., 2011). The differential capacity of NL1 and SC1 in this mixed-culture assay also likely accounts for the recovery of NLs, and not SCs, in a recent unbiased high-throughput screen for synaptogenic molecules (Linhoff et al., 2009). More rigorous spatiotemporal examination of terminal recruitment, similar to Lee et al. (2010), will thus be necessary to understand the different effects of NL1 and SC1 in the mixed-culture assay of artificial synaptogenesis.

Though a role for SC1 in developmental synaptogenesis has been clearly established both *in vitro* (Stagi et al., 2010; Fogel et al., 2011) and *in vivo* (Robbins et al., 2010), what this role is remains a provocative question. Continuous SC1 overexpression is necessary to maintain increased synapse densities in transgenic mice overexpressing SC1, suggesting that one role of SC1 may be to stabilize and maintain synaptic connections (Robbins et al., 2010). Such synapse stabilization could conceivably be achieved via receptor recruitment and synapse maturation (Hoy et al., 2009). Indeed, SC1 overexpression in immature neurons increases mini frequencies, consistent with an acceleration of synapse maturation that is occluded in more mature neurons (Sara et al., 2005). Further supporting this idea, we found that co-

overexpressing SC1 with NL1 precipitates NMDAR-mediated activity-dependent excitotoxicity, presumably through receptor recruitment to immature synaptic connections forged by NL1. While indirect, we find this evidence of cooperative NL1 and SC1 signaling compelling for two reasons. First, neither NL1 nor SC1 overexpression alone induced the pronounced neurodegeneration observed upon NL1/SC1 co-overexpression. Second, the neurodegenerative effect specifically required extracellular SC1 signaling and was thus likely the product of a synaptic mechanism. Our results thus contend multiple aspects of the proposed activity-dependent maturation model of NL1 function and instead support an alternative model whereby SC1 can mature new synaptic connections forged by NL1.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
DIV	day <i>in vitro</i>
GABA	γ -aminobutyric acid
Mini	spontaneous miniature postsynaptic current
NL	neuroligin
NMDAR	<i>N</i> -methyl-D-aspartate receptor
NRX	neurexin
RNAi	RNA-interference
SC	SynCAM

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Highlights

Neurologin-1 robustly recruits presynaptic terminals independent of synaptic activity

Neither gain- nor loss-of-function SynCAM1 perturbations impact terminal recruitment

Neurologin-1 is more potent than SynCAM1 in a mixed-culture assay of synaptogenesis

Neurologin-1/SynCAM1 co-overexpression triggers glutamatergic excitotoxicity

Our data suggest that SynCAM1 matures synaptic connections forged by neurologin-1

Figure 1. Dose-dependent relationship between NL1 expression and synapsin punctum densities. **A–C**, Neurons were transfected with distinct masses of a plasmid expressing HA:NL1 and processed for MAP2, HA, and synapsin immunostaining. Representative neurons from high overexpression (**A**; 500 ng expression vector), overexpression (**B**; 100–200 ng expression vector), and low overexpression (**C**; 20–50 ng expression vector) conditions are shown. Untransfected neighboring cells (identified by negative HA immunostaining) were also analyzed. Scale bars: 50 μm ; boxed regions of interest: 50 μm in length. **D**, Increased NL1 overexpression was consistently associated with increased synapsin punctum densities. **E**, Dendritic synapsin punctum densities were significantly correlated with the logarithm of exogenous NL1 expression (linear regression of log transformed mean HA:NL1 intensity, red line; $R^2=0.75$, $p<0.001$).

Figure 2.

Transsynaptic NL1 signaling mediates formation of immature synaptic connections. **A–F**, Neurons were transfected with plasmids expressing HA:NL1 and EGFP (**A,B**), EGFP (**C,D**), or HA:NLswap and EGFP (**E,F**) and processed for synapsin immunostaining (**A,C,E**; scale bars: 50 μ m; boxed regions of interest: 50 μ m in length) or electrophysiology (**B,D,F**). **G,H**, High NL1 overexpression significantly increased synapsin punctum densities (**G**) and dendritic spine densities (**H**) compared to EGFP control and NLswap expression conditions. **I**, NLswap expression significantly reduced the average mini frequency from baseline levels measured in EGFP controls. A strong trend existed for similar reduction in mini frequency with high NL1 overexpression ($p=0.078$). **J**, Both high NL1 overexpression and NLswap expression significantly reduced mini amplitudes compared to EGFP controls.

Figure 3.

NL1 initiates formation of immature synaptic connections independent of NMDAR-mediated activity. **A,B**, Neurons overexpressing HA:NL1 with EGFP were cultured normally (**A**) or were cultured under chronic NMDAR blockade with 100 μ M D-AP5 (**B**) until synapsin immunostaining. Scale bars: 50 μ m; boxed regions of interest: 50 μ m in length. **C,D**, Chronic NMDAR blockade did not significantly alter NL1-mediated gains in synapsin punctum densities (**C**), but did reduce NL1-mediated gains in dendritic spine densities (**D**).

Figure 4.

Validating the efficacy of SC1 knockdown using RNAi. HEK293FT cells were cotransfected with the FLAG:SC1 expression vector and the empty RNAi vector (lane 2), the RNAi vector expressing a control hairpin sequence (lane 3), or the RNAi vector expressing the designed hairpin targeting SC1 (lane 4). Protein was collected from cells 2 days post transfection, separated by SDS-PAGE, and immunoblotted for the FLAG epitope, with actin as a loading control. SC1 migrated as two bands near 70 and 100 kDa, as expected (Fogel et al., 2007). HEK293FT cells did not endogenously express the FLAG epitope (lane 1). The designed short-hairpin strongly reduced SC1 protein levels compared to empty vector and control hairpin conditions in two separate trials (representative blot shown). Arrow: artifact from membrane edge.

Figure 5.

SC1 perturbation does not alter terminal recruitment. **A–C**, Neurons were transfected with plasmids expressing FLAG:SC1 and EGFP (**A**), EGFP (**B**), or shSC1 and EGFP (**C**) and processed for synapsin immunostaining. Scale bars: 50 μm ; boxed regions of interest: 50 μm in length. **D,E**, Neither overexpression nor acute knockdown of SC1 significantly altered synapsin punctum densities (**D**) or dendritic spine densities (**E**).

Figure 6.

NL1 triggers greater recruitment of synapsin than does SC1 in the artificial synaptogenesis assay. **A,B**, HEK293FT cells expressing NL1 or SC1 (with cotransfection marker DsRed2) were cocultured with neurons for 1–2 days before synapsin immunostaining. Representative HEK293FT cells expressing NL1 (**A**) or SC1 (**B**) are shown. Scale bars: 25 μ m. Digital zoom: 2X. Resolution: 1024 $\times$ 1024. **C**, Quantification of the fractional HEK293FT cell area colocalized with suprathreshold synapsin immunofluorescence revealed that NL1-expressing cells triggers significantly greater synapsin recruitment than SC1-expressing cells.

Figure 7.

Combined NL1 and SC1 signaling induces neurodegeneration. **A,B**, Neurons were transfected with plasmids expressing HA:NL1 and EGFP (**A**) or HA:NL1, FLAG:SC1, and EGFP (**B**) and imaged 3–4 days later by EGFP fluorescence. 8 representative neurons are shown for each condition. Note the limited arborization, dendritic fragmentation, and swellings prevalent along axonal and dendritic processes in all neurons co-overexpressing NL1 with SC1 (**B**). Scale bars: 50 μ m.

Figure 8.

SC1 triggers NMDAR-mediated activity-dependent neurodegeneration at NL1-mediated synapses. **A,B**, Neurons were transfected with plasmids expressing HA:NL1 and EGFP (**A**) or HA:NL1, FLAG:SC1, and EGFP (**B**) and cultured under chronic NMDAR blockade (100 μ M D-AP5) until synapsin immunostaining. Note that chronic NMDAR blockade precluded all signs of neurodegeneration mediated by co-overexpression of SC1 with NL1. Scale bars: 50 μ m; boxed regions of interest: 50 μ m in length. **C,D**, During chronic inhibition of NMDARs by AP5, co-overexpression of SC1 with NL1 did not significantly alter synapsin punctum densities (**C**) or dendritic spine densities (**D**) from the NL1-overexpression condition.

Figure 9.

NMDAR-mediated activity-dependent neurodegeneration triggered by co-overexpression of SC1 with NL1 specifically requires transsynaptic SC1 signaling. **A,B**, Neurons overexpressing NL1 alone (**A**) or with SC1 mutant SCΔIg (**B**) were processed for synapsin immunostaining. Note expression of SCΔIg with NL1 did not induce any signs of neurodegeneration. Scale bars: 50 μm; boxed regions of interest: 50 μm in length. **C,D**, Expression of SCΔIg with NL1 did not significantly alter synapsin punctum densities (**C**) or dendritic spine densities (**D**) from NL1-overexpression levels. Neuroligin-1 robustly recruits presynaptic terminals independent of synaptic activity. Neither gain- nor loss-of-function SynCAM1 perturbations impact terminal recruitment. Neuroligin-1 is more potent than SynCAM1 in a mixed-culture assay of synaptogenesis. Neuroligin-1/SynCAM1 co-overexpression triggers glutamatergic excitotoxicity. Our data suggest that SynCAM1 matures synaptic connections forged by neuroligin-1.