Alternative Splicing of Neuroligin Regulates the Rate of Presynaptic Differentiation

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Neuroligins (NLGs) and Neurexins (NRXs) are important adhesion molecules that promote synapse formation. Multiple splice variants of NLG and NRX exist, but their specific functions are unclear. Here we report that a surrogate postsynaptic cell expressing full-length NLG-1 triggers slow presynaptic differentiation in a contacting axon. In contrast, a version of NLG-1, which lacks insert B (NLG-1ΔB), induces rapid presynaptic differentiation, reaching the rate seen at native neuronal synapses. We show that this acceleration is attributed to the removal of the N-linked glycosylation site within insert B. NLG-1ΔB also increases synaptic density at neuro-neuronal synapses more than does full-length NLG-1. Other postsynaptic adhesion proteins, such as N-cadherin, EphB2, and SynCAM-1, alone or in combination with full-length NLG-1, do not trigger fast differentiation, suggesting that rapid presynaptic differentiation depends on a unique interaction of NLG-1ΔB with axonal proteins. Indeed, we find that NLG-1ΔB recruits more axonal α -NRX. Our results suggest that the engagement of α -NRX is a key to rapid induction of synapses at new sites of axo-dendritic contact.

Introduction

During the development of synapses in the CNS, dynamic protrusions from axons and dendrites extend and retract on a timescale of minutes, probing the environment for targets (Portera-Cailliau et al., 2003). Once appropriate contacts are made, synapse formation must quickly follow to stabilize these transient adhesive connections (Niell et al., 2004; Ruthazer et al., 2006). This requires fast accumulation of adhesion molecules, rapid transport, and accurate deposition of synaptic proteins to these locations. On the presynaptic side, many synaptic proteins are packaged in complexes for transport. At least two classes of transport packets have been identified: clear circular vesicles ~50 nm in diameter, which likely correspond to synaptic vesicles or their precursors, and 80 nm dense core vesicles, named Piccolo transport vesicles (PTVs) because they carry the cytomatrix active zone proteins Piccolo and Bassoon (BSN) (Zhai et al., 2001; Shapira et al., 2003) as well as other proteins present in the active zone (Garner et al., 2006). Both of these complexes can arrive at new synapses within 20–30

min of physical contact between axons and dendrites, leading to the formation of new synapses that release neurotransmitter in an activity-dependent manner within 1 h of contact (Ahmari et al., 2000; Friedman et al., 2000; Bresler et al., 2004).

Multiple adhesion molecules are present at synapses (Dalva et al., 2007), although single classes of postsynaptic adhesion molecules are sufficient to induce presynaptic differentiation at sites of pre-post contact. Five postsynaptic adhesion protein families, Neuroligin (NLG) (Scheiffele et al., 2000; Dean et al., 2003), synaptic cell adhesion molecule (SynCAM) (Biederer et al., 2002), Netrin-G ligand-2 and -3 (NGL-2, NGL-3) (Kim et al., 2006; Woo et al., 2009), EphB2 (Kayser and Dalva, 2005), and LRRTM2 (de Wit et al., 2009; Ko et al., 2009a), when presented by a non-neuronal cell, trigger presynaptic differentiation. However, the dynamics of the interactions between these adhesion molecules and their presynaptic cognates as well as the subsequent nucleation of the presynaptic transmitter release machinery have not been fully determined, although existing data suggest that the interaction between Neuroligin and its ligand β -Neurexin (β -NRX) (Ichtchenko et al., 1995) are fast enough for the fast synapse formation observed in neurons (on the order of minutes) in both non-neuronal S2 and PC12 cells (Nguyen and Südhof, 1997; Dean et al., 2003) and between β -NRX-expressing HEK293 cells and NLG-bearing supported bilayers (Pautot et al., 2005).

To address these issues, we used an assay in which postsynaptic neurons are replaced by a surrogate cell: a HEK293 cell expressing one or more postsynaptic adhesion proteins (Scheiffele et al., 2000; Biederer and Scheiffele, 2007). This enabled us to define the molecular constituents of the cell–cell interaction and the time and location of contacts. We compared the ability of different postsynaptic adhesion molecules to rapidly (within 1 h of contact) induce synapse formation. Strikingly, among NLG-1, NLG-1ΔB (a splice variant of NLG-1 missing an exon of 9 aa), SynCAM, EphB2, NGL-2, and N-Cadherin, only NLG-1ΔB was able to recruit Bassoon to new

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contacts and induce functional presynaptic terminals. We find that α -NRX, an important component for NLG-mediated presynaptic differentiation (Ko et al., 2009b), is preferentially concentrated at contacts with NLG-1 Δ B-expressing cells, consistent with biochemical evidence that absence of insert B enhances α -NRX binding (Boucard et al., 2005). Conversely, a mutation removing an N-linked glycosylation site in insert B known to impair α -NRX binding (Boucard et al., 2005) induced rapid presynaptic differentiation. Finally, overexpression of NLG-1 Δ B increased synaptic density to a higher degree than NLG-1 (and higher than control vector). Together our data show that NLG-1 Δ B is adapted for the fast synapse induction seen at new axo-dendritic contacts. This implies that alternative splicing of NLG plays a role in regulating the rate of synapse formation.

Materials and Methods

All chemicals were purchased from Sigma unless otherwise noted.

DNA constructs and antibodies

The expression plasmid for Neuroligin-1 was described previously (Scheiffele et al., 2000). Monomeric cyan (mCFP), green (mGFP), and yellow (mYFP) fluorescent protein (Zacharias et al., 2002) or monomeric red fluorescent protein (mRFP) (Campbell et al., 2002) fragments were amplified by PCR and inserted into a unique Sall site in vesicular stomatitis virus glycoprotein peptide-tagged β -NRX-1 just after the LNS domain. The fluorescent protein (FP) was placed at the same site in FLAG-tagged α -NRX-1 (Boucard et al., 2005) by swapping the ectodomain from FP-tagged β -NRX-1. For NLG-1-mRFP constructs, mRFP was inserted at an engineered SacII site between the AChE-like domain and the transmembrane domain. The NLG-N303S mutation was made by changing AAT to TCT with the QuikChange Site-Directed Mutagenesis kit from Stratagene. SynCAM-1, GFP-tagged Bassoon (GFP:BSN95-3938), Synaptophysin (SYP-GFP), calcium/calmodulin-dependent serine kinase (GFP-CASK), EphB2, and NGL-2 were provided by Dr. Südhof (Stanford University, Palo Alto, CA), Dr. Gundelfinger (Leibniz Institute for Neurobiology, Magdeburg, Germany) (Shapira et al., 2003), Dr. Kaether (Leibniz Institute for Age Research, Jena, Germany) (Kaether et al., 2000), Dr. Reichardt (University of California, San Francisco, CA) (Bamji et al., 2003), Dr. Irie (Sanford-Burnham Medical Research Institute, La Jolla, CA), and Dr. Kim (Korea Advanced Institute of Science and Technology, Daejeon, South Korea) (Kim et al., 2006), respectively. SYP-YFP was made by replacing the GFP with YFP. Postsynaptic density-95 (PSD-95)-GFP was provided by Dr. Lu Chen (University of California, Berkeley, CA), and GFP was replaced with mRFP to make the red version.

All four short hairpin RNAs (shRNAs) were integrated into one pSuper-Retro-GFP plasmid (Oligoengine) according to the cloning method described previously (Stove et al., 2006). α -NRX-1 shRNA was directed against residues 2402–2420 (GenBank accession number NM_021767) with the sense sequence as follows: 5'-GCTATAACCTCAATGATAA-3'. α -NRX-2 and α -NRX-3 shRNAs were based on the target sequences of small interfering RNAs from Dharmacon: D-096072-1 against rat (*Rattus norvegicus*) α -NRX-2 (GenBank accession number NM_053846); and D-096239-03 and D-096239-04 against rat α -NRX-3 (GenBank accession number NM_053817).

Anti-PSD-95 antibody (7E3-1B8) and anti-Synapsin-I antibody are from Affinity BioReagents and Millipore Bioscience Research Reagents, respectively. Anti-Bassoon antibody (SAP7F407) is from Stressgen or Abcam. Anti-FLAG antibody is from Sigma.

Neuronal cell culture and transfection

Rat (*R. norvegicus*) hippocampal neurons were dissociated from embryonic rats of both genders at embryonic day 18 (E18) to E19 and plated at 500–1000 cells/mm² on 12 mm coverslips (Carolina Biologicals) in MEM (Invitrogen) supplemented with 20 mM D-glucose, 2% B27, 5% fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin (all from Invitrogen), and Serum Extender (BD Biosciences). Neurons were transfected between days 5 and 10 in culture with a modified method of calcium phosphate precipitation (Xia et al., 1996). In brief, for each 12 mm coverslip, 2 μ g of DNA was diluted in 15 μ l of 250 mM CaCl₂ solution and mixed with 15 μ l of

HEPES-buffered saline (274 mM NaCl₂, 10 mM KCl, 1.4 mM Na₂HPO₄, 15 mM glucose, and 42 mM HEPES). At 30 min later, the mixture was combined with 2 ml of serum free culture media (MEM with B27, L-glutamine, and penicillin/streptomycin), added to neuronal cultures in a 35 mm dish, and incubated for 30 min to 2 h. The culture was then washed with calcium-free PBS three times, and the original culture media were replaced. Nucleofection (Lonza Cologne AG) was performed according to the manual of the supplier. In brief, 2 μ g of DNA was mixed with 2×10^6 freshly extracted mixed neurons/glia in 100 μ l of Nucleofector solution and then electroporated with the Nucleofector Program O-003. The mixture was then mixed with 500 μ l of culture media and incubated for 20 min before plating.

Image acquisition

One to 2 d after transfection, hippocampal neurons were incubated in modified Tyrode's saline solution (in mM: 115 NaCl, 2.8 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.3–7.4). HEK293 cells were also used 1–2 d after transfection and dissociated from the culture plate by enzyme-free dissociation media (Specialty Media/Millipore). Transfected HEK293 cells were then manipulated into contact with axons by controlled flow through a glass pipette. Time-lapse images were acquired on a Carl Zeiss LSM510 confocal microscope. The first frame was taken $\sim$ 1 min after the HEK293 cell landed on the axon as a result of the delay of focusing and switching to the time-lapse mode. Each frame was an average of two scans, and the interval between frames was 60–150 s depending on the experiments. Laser power, photomultiplier gain, and filter sets were selected to minimize bleaching and bleed-through between channels (in nm: CFP, excitation 458, emission 470–500; GFP, excitation 488, emission 500–550; YFP, excitation 514, emission 530–560; RFP, excitation 543, emission 565–615). Simultaneous imaging of pDisplay or CD8-YFP and FP-tagged NRXs was performed on a Carl Zeiss LSM 5 Live confocal microscope with a similar setting (in nm: CFP, excitation 440, emission 445–500; YFP, excitation 488, emission 530–590; RFP, excitation 533, emission 565 long pass).

FM staining

FM 4-64 [N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl) hexatrienyl]pyridinium dibromide (Invitrogen) was loaded into neurons by a 2-min high-K (in mM: 71.5 NaCl, 50 KCl, 2 CaCl₂, 2 MgCl₂, 20 HEPES, and 20 glucose, pH 7.3–7.4) depolarization in the presence of 15 μ M FM 4-64, washed with normal saline solution (in mM: 119 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 20 HEPES, and 20 glucose, pH 7.3–7.4) for 15 min, and unloaded by a 2-min 90-mM KCl (in mM: 31.5 NaCl, 90 KCl, 2 CaCl₂, 2 MgCl₂, 20 HEPES, and 20 glucose, pH 7.3–7.4) depolarization. In some cases, 1 mM Advasep-7 (CyDex) was added in the washing solution to facilitate the washout of extracellular FM 4-64. The dye was excited at 514 nm, and a 650 nm long-pass filter was used to collect the signal.

Image analysis

All images are analyzed in Matlab (MathWorks) using custom scripts.

Time-lapse experiments. Offline line scan was acquired by drawing a 10-pixel-wide line along the axon, so each pixel in the line scan is an average of 10 pixels perpendicular to the line. Such measure was taken to minimize movement artifacts. Total intensity under the HEK293 cell was measured by summing the pixel intensity value within regions of interest defined by the contour of the surrogate postsynaptic cells. The average background was then subtracted from this sum. Accumulation plots in supplemental Figure 2 (available at www.jneurosci.org as supplemental material) were made by setting the initial value at 0% and the final value at 100%.

Immunostaining. Because individual Bassoon and Synapsin (SYN) puncta became difficult to resolve at high density, we used coverage as an indicator for number of associated synapses. First, a binary mask was generated based on fluorescence of the HEK293 cell to cover the whole HEK293 cell, and a second mask was then made by thresholding the Bassoon or Synapsin fluorescence within the first mask. The coverage percentage was calculated by dividing the area of the second mask by the first one.

Relative density. To calculate the relative density of NRXs at the cell contacts, first a binary mask was generated by thresholding the pDisplay-YFP or CD8-YFP (plasma membrane markers) image. Then within this mask, we calculate the density of NRXs by dividing the intensity of NRXs by the intensity of the plasma membrane marker, which accounts for any heterogeneous membrane protein distribution, assuming the plasma membrane marker is not preferen-

Table 1. Fitting models comparison

Model	Separate regressions	Three parallel lines	Three rotated lines	NLG-ΔB = NLG	NLG = CD8	Single line
Number of parameters	6	4	4	3	3	2
SSE	119.51	121.25	124.52	128.40	128.44	152.70
F	0.90			7.43	7.48	16.34
p value	40.8%			0.7%	0.7%	<0.1%

NLG-ΔB = NLG: NLG-1 and NLG-1ΔB transfected neurons are considered drawn from the same population, whereas CD8-YFP transfected ones are different. NLG = CD8: NLG-1 and CD8-YFP transfected neurons are considered drawn from the same population, whereas NLG-ΔB transfected ones are different. Single line: All three groups belong to the same population. All *F* tests and corresponding *p* values are compared with the parallel-lines model, with the listed models as the null hypotheses.

tially distributed to any particular region. Finally, the relative density is the average density at the cell contact divided by the average density elsewhere on the cell.

Density of synapses. Images of transfected neurons were turned into binary images by passing through a threshold. The soma was removed from the binary images and then skeletonized, with short branches (<6 μm) removed. The resulting images were the backbones of the dendritic trees in which the total lengths can be measured. Particle tracking routines from D. Blair (Georgetown University, Washington, DC) and E. Dufresne (Yale University, New Haven, CT) (<http://www.physics.georgetown.edu/matlab/>) were used to locate individual Bassoon puncta. In brief, images of Bassoon immunostaining are first filtered spatially to minimize background. Then the filtered images are fitted with a two-dimensional Gaussian curve to obtain the center coordinates. Any Bassoon punctum within 1 μm of the dendrites is considered to be colocalized.

RNA interference

Images of transfected neurons cocultured with HEK293 cells were analyzed manually. LSM Image Browser (Carl Zeiss) was used to view the images and to identify Bassoon puncta at the contacts between HEK293 cells and transfected neurons. The lengths of the contacts were traced by hand and measured with the LSM Browser. Synaptic density was calculated by dividing the number of Bassoon puncta by the length of the contact. To confirm the analysis and avoid bias, the analysis was repeated by another experimenter blind to the identity of each experimental group.

Statistical analysis

All graphs are expressed as average ± SEM. Statistical tests are indicated in the figure legends. Monoexponential recovery curves are fitted to BSN, SYN, and α-NRX accumulation using the following function:

$$y_i = y_{\infty}(1 - e^{-\frac{t_i}{\tau}}) + \varepsilon_i,$$

where y_i are the coverage at time t_i , y_{∞} is the maximal accumulation amount, τ is the time constant, and ε_i are the residuals. The fitting was done in Matlab using its Curve Fitting Toolbox with nonlinear least square method, starting from $(y_{\infty}, \tau) = (0.5, 2)$. The initial rate of accumulation is

the derivative of the exponential curve at $t = 0$, which equals to $\frac{y_{\infty}}{\tau}$.

For the analysis of NLG-1 and NLG-1ΔB overexpression in neurons, two independent transfections were made and analyzed separately. A total of 39–50 neurons from four coverslips per experimental condition were imaged and measured to obtain the synapse density and the total number of synapses in each field of view. We then followed the standard method of analysis of covariance (ANCOVA), in which synaptic densities on transfected neurons are linearly regressed to total number of synapses of each frame and categories, following the function below:

$$y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \varepsilon_i,$$

where y_i is the synapse density of transfected neurons, X_{i1} is the total number of synapses in the same visual field,

$$X_{i2} = \begin{cases} 1 & \text{if neuron is transfected with NLG-1} \\ 0 & \text{otherwise} \end{cases},$$

$$X_{i3} = \begin{cases} 1 & \text{if neuron is transfected with NLG-1ΔB} \\ 0 & \text{otherwise} \end{cases}.$$

β_0 , β_1 , β_2 , and β_3 are fitted coefficients, and ε_i are the residuals.

The fitted function represents three parallel lines that all have the same slope α but with different intercepts (CD8-YFP control = β_0 , NLG-1 = $\beta_0 + \beta_2$, NLG-1ΔB = $\beta_0 + \beta_3$). The parallel lines model is then compared with the other restrictive models in which two categories are considered the same and the other different, or all three are considered the same. ANOVA on the sum of squared residuals showed that the three-parallel-lines model provides significantly more explanation power than the other restrictive models. The parallel lines model is also compared with the model in which each group is linear-regressed separately or the rotated lines model in which slopes differ between each group but the intercept is assumed to be the same. Additional two parameters used in separate regressions did not provide significant explanation power ($p = 40.8\%$), whereas three-rotated-lines model decreases the explanation power (Table 1).

Results

Neuroligin-1ΔB induces rapid synapse formation

Although several classes of postsynaptic adhesion molecules are known to trigger presynaptic differentiation, it is unclear whether any of these molecules is sufficient to achieve the rapid synapse formation necessary to stabilize transient contacts (Niell et al., 2004; Ruthazer et al., 2006). We compared the rates of induction of presynaptic terminals in cultured hippocampal neurons at new sites of contact with surrogate postsynaptic cells in the coculture hemisynapse formation assay (Scheiffele et al., 2000). The surrogate postsynaptic cells were HEK293 cells expressing one or more of the six different adhesion molecules. The adhesion molecules included EphB2 (Kayser and Dalva, 2005), SynCAM (Biederer et al., 2002), NGL-2 (Kim et al., 2006), two alternative splice products of NLG-1, the full-length version, and a version lacking insert B (NLG-1ΔB) (Ichtenko et al., 1995; Scheiffele et al., 2000), and N-cadherin (Fannon and Colman, 1996; Togashi et al., 2002; Abe et al., 2004). The adhesion molecules were transfected, singly or in pairs, into HEK293 cells, and the HEK293 cells were dropped onto E18–E19 hippocampal neurons that had been developing *in vitro* for 7 d (7 DIV). Unlike previous studies in which synapse formation on HEK293 cells was assayed 24 h or longer after surrogate–neuron contact, our cocultures were fixed and stained with antibodies against the active zone protein Bassoon 1 h after contact. Strikingly, compared with CFP-expressing control HEK293 cells, only HEK293 cells expressing NLG-1 lacking insert B induced a significant accumulation of BSN 1 h after contact (Fig. 1A,B).

We next asked whether the newly induced sites of Bassoon accumulation were competent for transmitter release. This was done by determining whether membrane depolarization would stimulate the FM dye uptake that is associated with activity-dependent recycling of synaptic vesicles. We found that, 1 h after contact with NLG-1ΔB-expressing HEK293 cells, axons have significantly more FM 4-64-positive puncta (7.9 ± 0.9 puncta per cell, $n = 6$) at the sites of the contact than those axons contacting HEK293 cells expressing NLG-1 (2.8 ± 0.7 puncta per cell, $n = 5$, $p < 0.05$, Student's *t* test) (Fig. 1C). These observations indicate that NLG-1ΔB has a special ability

Figure 1. HEK293 cell expressing NLG-1ΔB induces rapid formation of presynaptic terminal on neurons. **A**, Accumulation of endogenous Bassoon (red) after 1 h of contact with HEK293 cells expressing different adhesion molecules. CFP (cyan) was cotransfected with untagged adhesion molecules. **B**, Bassoon density was significantly higher under HEK293 cells expressing NLG-1ΔB than under HEK293 cells expressing either NLG-1 alone or another adhesion protein alone or in combination with NLG-1 (Dunnett's test, $n = 10$ fields per group, $*p < 0.05$). **C**, HEK293 cells expressing NLG-1 or NLG-1ΔB tested for the ability to induce functional synaptic vesicle release sites within 1 h as measured by the uptake of FM 4-64. More FM 4-64 puncta (red) were colocalized with HEK293 cells expressing NLG-1ΔB than with those expressing NLG-1 alone. On average, NLG-1-expressing HEK293 cells have 2.8 ± 0.7 FM puncta per cell, and NLG-1ΔB-expressing cells have 7.9 ± 0.9 FM puncta per cell, significantly more than the NLG-1 group ($n = 5$ and 6 respectively, $p < 0.05$, Student's t test). **D**, A typical example of the time course of new synapse appearance [as gauged by endogenous BSN (green) and SYN (red) accumulation] around HEK293 cells (white dashed lines) expressing NLG-1ΔB (representative images in the top panel; red triangles in the bottom graphs) or NLG-1 (representative images in the middle panel; blue squares in the bottom graphs). Scale bar, $10 \mu\text{m}$. Average values graphed at bottom, blue and red lines are monoexponential fits to data. Because individual BSN and SYN puncta become difficult to resolve at the high density reached at the later time points, we compared the percentage of the HEK293 cell surface covered by BSN or SYN. The formation of BSN and SYN puncta on NLG-1 cells lags behind that seen on NLG-1ΔB cells by 3–8 h. **E**, The initial accumulation rates of BSN and SYN in three independent experiments. NLG-1ΔB always induced rapid synapse formation compared with NLG-1.

to induce the rapid recruitment of the presynaptic machinery and the formation of functioning presynaptic nerve terminals.

When the axonal accumulation of synaptic vesicles and PTVs at sites of HEK293 cell contact was examined over 24 h by immunostaining for native SYN and BSN, we found that, although the amount of recruitment by NLG-1 and NLG-1ΔB eventually converges, NLG-1ΔB recruits SYN and BSN more rapidly (Fig. 1D). Single exponential fits indicated that the recruitment of SYN and BSN induced by NLG-1ΔB occurred over a similar time course, with a time constant of ~ 3 h ($\tau_{\text{SYN}} = 2.80$ h, $n = 7$ –10 cells per time point; $\tau_{\text{BSN}} = 2.96$ h, $n = 7$ –10 per time point), whereas the recruitment of BSN by the full-length NLG-1 was more than twofold slower ($\tau = 6.52$ h, $n = 7$ –10 per time point), and the recruitment of SYN by the full-length NLG-1 was almost fourfold slower ($\tau = 11.11$ h, $n = 7$ –10 cells per time point). The experiments were repeated in two separate sets of cell cultures and transfections, yielding an average initial accumulation rate (y_{∞}/τ) of BSN and SYN, as shown in Figure 1E. In all cases, NLG-1ΔB always promoted faster presynaptic differentiation than did NLG-1. Together, the results demonstrate that, among all the adhesion molecules tested (NLG-1, NLG-1ΔB, N-cadherin, EphB2, SynCAM, and NGL-2), NLG-1ΔB induces the fastest presynaptic differentiation.

We next followed synapse formation in real time using time-lapse microscopy to focus on axons before and after contact with NLG-1ΔB-expressing HEK293 cells. To do this, we transfected the neurons at 7–10 DIV with either an N-terminal GFP fusion of

Bassoon (GFP–Bassoon) or a C-terminal YFP fusion of the highly restricted synaptic vesicle protein Synaptophysin (Synaptophysin–YFP). One or 2 d later, axons were imaged for a control frame, and then HEK293 cells expressing NLG-1ΔB were positioned into contact with them and imaging was continued. In 25% (5 of 20 experiments) of the axons imaged in this way, contact with the NLG-1ΔB-expressing HEK293 cell triggered the recruitment of GFP–Bassoon puncta within 15–30 min (Fig. 2A), comparable with what has been observed at new neuro-neuronal contacts (Friedman et al., 2000). The higher incidence with which we observed the accumulation of native Bassoon in the 1 h endpoint antibody labeling experiments as opposed to GFP–Bassoon in the time-lapse experiments may reflect the fact that the native Bassoon was usually imaged in multiple axons that came into contact with each HEK293 cell, whereas in the time-lapse experiments, GFP–Bassoon was followed in only one contacting axon at a time. A second factor is that the time-lapse experiments on GFP–Bassoon were performed at a lower temperature ($\sim 25^{\circ}\text{C}$) under atmospheric conditions compared with the physiological temperature (37°C) and 5% CO_2 used in the antibody-labeling experiments.

Consistent with what has been described for the dynamics of the synaptic vesicle protein VAMP2 at neuro-neuronal synapses (Ahmari et al., 2000), Synaptophysin–YFP accumulated rapidly in small puncta under the NLG-1ΔB-expressing HEK293 cells. As with GFP–Bassoon, the newly recruited Synaptophysin–YFP was seen in some of the contacts (8 of 15). Also, as with GFP–Bassoon,

Figure 2. NLG-1 Δ B on HEK293 cell rapidly clusters axonal PTVs and SVs within the first hour after contact. **A**, Time-lapse imaging of GFP–Bassoon recruitment to contact with NLG-1 Δ B-expressing HEK293 cell (supplemental Movie 1, available at www.jneurosci.org as supplemental material) within ~ 15 min after contact (white arrows in images on left, central streak on line-scan depiction on right). Images taken 1 and 3 min after contact and then once every 3 min thereafter. **B**, Time-lapse imaging of SYP–YFP recruitment to contact with NLG-1 Δ B-expressing HEK293 cell (supplemental Movie 2, available at www.jneurosci.org as supplemental material) within ~ 30 min after contact (white arrows in images on left, central streak on line-scan depiction on right). Image acquired once every minute. HEK293 cell expressing NLG-1 Δ B is outlined with a white circle (left). Line scan along axon (right) taken between yellow arrows indicated in top image (left). Dotted timeline in line scan indicates HEK293 cell boundary. Scale bar, 10 μ m.

when Synaptophysin–YFP puncta appeared their fluorescence increased gradually (Fig. 2B). The average intensity of new SYP–YFP puncta at NLG-1 Δ B contacts was lower ($69 \pm 14\%$, $n = 8$) than that seen at preexisting stable puncta. We conclude that both Synaptophysin-containing synaptic vesicles and Bassoon-containing PTVs arrive rapidly to new presynaptic terminals induced by NLG-1 Δ B.

Neuroligin-1 Δ B has an increased affinity to α -Neurexin in neurons

Biochemically, the main difference between NLG-1 Δ B and full-length NLG-1 is that NLG-1 Δ B binds to both α - and β -NRXs, whereas NLG-1 only binds to β -NRX (Boucard et al., 2005). Moreover, it was shown recently that a long (48 h) period of contact with a surrogate postsynaptic cell expressing NLG-1 Δ B in hemisynapse formation that primarily depends on α -NRX in the neuron (Ko et al., 2009b). This suggested to us that α -NRX may

also play a role in the rapid induction of presynaptic differentiation by NLG-1 Δ B.

We asked whether α -NRX in the neuron is preferentially delivered to sites of contact with NLG-1 Δ B-expressing HEK293 cells. To do this, we generated FP-tagged versions of α - and β -NRXs by inserting a monomeric fluorescent protein between the last LG/LNS domain and transmembrane domain (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). The tagged proteins were shown to retain the ability of untagged NRXs to undergo heterophilic adhesion with NLG-1 and NLG-1 Δ B in the following experiments. First, when α -NRX–mYFP- or β -NRX–mCFP-expressing HEK293 cells were brought into contact with NLG-1 expressed in two separate HEK293 cells and the cells were brought into contact, α -NRX–mYFP and β -NRX–mCFP concentrated at cell–cell junctions [as shown for β -NRX–mCFP in supplemental Fig. 1B (available at www.jneurosci.org as supplemental material) and for α -NRX–

Figure 3. Preferential recruitment of α -NRX to NLG-1 Δ B at HEK293–HEK293 contacts. **A**, Quantification of accumulation in 24 h of α - or β -NRX–mCFP in HEK293 cell at contact with HEK293 cell expressing NLG-1, NLG-1 Δ B, or NLG-1 (N303S). pDisplay–YFP labeling of the plasma membrane was used to calculate α - or β -NRX–mCFP density per unit membrane. Density of α - or β -NRX–mCFP at the contact was then normalized to average density at non-contacting areas (for details, see Materials and Methods). α -NRX accumulates strongly at NLG-1 Δ B and NLG-1 (N303S) contacts but weakly at NLG-1 contacts (Dunnett's test, $*p < 0.05$). Note that the relative density of α -NRX at NLG-1 contacts is ~ 1.2 (i.e., 120% of the average density on the cell), indicating that NLG-1 recruits a small amount of α -NRX ($p < 0.05$, one sample t test vs 100%). **B**, Comparison of recruitment of α -NRX–mYFP (green) and β -NRX–mCFP (cyan) coexpressed in one HEK293 cell to site of contact with HEK293 cell expressing either NLG-1–mRFP (red) or NLG-1 Δ B (cotransfected with mRFP, colored in red) with which it is cocultured for 24 h. Scale bars, 10 μ m. **C**, Fraction of α -NRX–mYFP and β -NRX–mCFP at contact sites. NLG-1 Δ B (magenta symbols) recruits majority of both α -NRX and β -NRX and has similar efficacy for two NRXs (symbols lie near unity line). NLG-1 (blue symbols) recruits β -NRX preferentially (symbols cluster near ordinate). Recruitment of α -NRX is much stronger by NLG-1 Δ B than by NLG-1.

mYFP in Fig. 3B]. To quantify the recruitment, we measured the relative density of NRXs at the cell–cell junction (i.e., the average density at the junction divided by the average density elsewhere on the cell). pDisplay–YFP, a plasma membrane marker, was used to control for any unrelated heterogeneous membrane protein distribution, so the density of NRXs was normalized to the density of pDisplay–YFP. As expected, both NLG-1 and NLG-1 Δ B recruited β -NRX equally well, with the relative density of β -NRX–mCFP at the junction being ~ 2 (i.e., the normalized fluorescence of β -NRX–mCFP at the junction was two times higher than elsewhere on the cell) (Fig. 3A). In contrast, the relative density of α -NRX–mCFP at the NLG-1 Δ B junctions was

2.08 ± 0.22 ($n = 10$), significantly higher than at the NLG-1 junctions (1.19 ± 0.08 , $n = 10$), indicating that there is a preference of α -NRX–mCFP for NLG-1 Δ B. To test whether such a preference remains even when both types of FP-tagged NRXs are present in the same cell, as is the case in neurons, we coexpressed α -NRX–mYFP and β -NRX–mCFP in the same HEK293 cell and brought this cell into contact with another HEK293 cell bearing either NLG-1 or NLG-1 Δ B (Fig. 3B). The preference could be visualized by plotting the fraction of α -NRX–mYFP at the junction versus that of β -NRX–mCFP (Fig. 3C). NLG-1 Δ B recruited both α - and β -NRX (thus the magenta points in Fig. 3C approximately follow the 1:1 ratio diagonal line), whereas NLG-1 recruited mostly β -NRX (thus the blue points in Fig. 3C deviate toward the ordinate). These results confirmed that insertion of fluorescent protein in α - and β -NRXs did not modify their affinities for NLG-1 and NLG-1 Δ B.

Having found that NLG-1 Δ B recruits α -NRX more potently than does full-length NLG-1 at HEK293–HEK293 contacts, we next asked whether the same would be true at contacts between an NLG-1-presenting HEK293 cell serving as a postsynaptic surrogate and an axon expressing the panoply of other neuronal proteins. We coexpressed α -NRX–mCFP in neurons along with CD8–YFP (a plasma membrane marker) as a reference and examined sites of contact with HEK293 cells expressing NLG-1 Δ B or NLG-1 over 24 h. We observed a stronger α -NRX–mCFP accumulation at sites of contact with HEK293 cells expressing NLG-1 Δ B than with HEK293 cells expressing NLG-1. The difference was evident at 1 h (relative density for NLG-1 Δ B/NLG-1, 3.33 ± 0.43), 10 h (relative density, 3.69 ± 0.48), and 24 h (relative density, 3.75 ± 0.81) after contact (Fig. 4A). Although α -NRX–mCFP was also recruited to sites of contact with NLG-1 expressing HEK293 cells, its concentration was significantly lower (relative density: 1.38 ± 0.18 at 1 h, 1.41 ± 0.15 at 10 h, and 2.00 ± 0.31 at 24 h) (Fig. 4B, $p < 0.05$). In contrast, the concentration of β -NRX–mCFP did not differ between neurons contacting NLG-1 and those contacting NLG-1 Δ B, although no additional increase was observed after 1 h as well (Fig. 4C). Notice that no additional increase of α -NRX–mCFP was observed beyond 1 h after contact, indicating that its accumulation reaches maximum within 1 h, preceding the recruitments of BSN and SYN (Fig. 4D).

Our observations at HEK293–HEK293 and neuron–HEK293 contacts generally agree with previous work on the interaction of the soluble ectodomains of NRX and NLG (Ichtchenko et al., 1995; Boucard et al., 2005). Although these previous studies suggested that α -NRX and full-length NLG-1 do not interact, our results indicate that they have a weak interaction when they are presented on cell membranes.

We next asked whether the unique potency of NLG-1 Δ B could be attributed to a more rapid recruitment of axonal α -NRX than β -NRX to the contact site. To address this, we performed time-lapse imaging of both FP-tagged α -NRX and β -NRX in axons before and after contact with HEK293 cells expressing NLG-1 or NLG-1 Δ B. When such neurons were monitored for 1 h after contacting HEK293 cells expressing NLG-1, the accumulation of α -NRX–mCFP was always less than that of β -NRX–mYFP ($\alpha/\beta = 0.81 \pm 0.05$, $n = 5$) (Fig. 5A,B). However, when the transfected neurons were in contact with HEK293 cells expressing NLG-1 Δ B, the accumulation of α - and β -NRX was similar ($\alpha/\beta = 0.96 \pm 0.10$, $n = 5$) (Fig. 5A,B) and significantly different from the case of NLG-1 (paired t test, $p < 0.05$) (Fig. 5B). These data indicated that the removal of insert B in NLG-1 increases

Figure 4. Preferential recruitment of α -NRX to NLG-1 Δ B at HEK293–neuron contacts. Recruitment of axonal NRX to contact with NLG-expressing HEK293 cell. **A**, Representative examples of axonal α -NRX–mCFP accumulation at contact with HEK293 cell expressing NLG-1 (top) or NLG-1 Δ B (bottom) 1, 10, and 24 h after contact. Color map is set with average density of α -NRX–mCFP at noncontacting area to 1. Cotransfected CD8–YFP labeling of plasma membrane was used to calculate density (see Fig. 3 and Materials and Methods). **B**, Mean $\pm$ SEM for the dataset ($n = 7$ –10) shown in **A**. At all time points tested, α -NRX–mCFP concentrated at NLG-1 Δ B contacts (red triangles) significantly more than at NLG-1 contacts (blue squares) ($*p < 0.05$, Student's t test). The density of α -NRX–mCFP is higher at the contact site than in the surrounding area even for NLG-1. No additional increase of α -NRX–mCFP density was seen after 1 h. Red and blue lines represent single exponential fits. **C**, Mean $\pm$ SEM ($n = 7$ –10) for accumulation of axonal β -NRX–mCFP at contacts with HEK293 cells expressing NLG-1 (blue squares) or NLG-1 Δ B (red triangles) using the same method as in **A**. The two versions of NLG are equipotent at recruiting β -NRX, whose accumulation is maximal by 1 h after contact. **D**, Time course of accumulation of axonal proteins at site of contact with HEK293 cell expressing NLG-1 Δ B (solid lines) or NLG-1 (dashed lines). Normalized monoexponential fits to accumulation of BSN (red) and SYN (green) (from Fig. 1D, bottom) and α -NRX (blue, from **B**) are superimposed. α -NRX reaches plateau in ~ 1 h at both NLG-1 Δ B and NLG-1 contacts, although more accumulates at NLG-1 Δ B contacts (see **B**), and this is associated with faster recruitment of BSN and SYN. Also note that the time course for BSN recruitment overlaps that of SYN at NLG-1 Δ B contacts.

α -NRX concentration at HEK293–neuron contacts but has no effect on β -NRX concentration.

So far, we have shown that contact with a cell presenting NLG-1 Δ B induces a more potent local accumulation of active zone proteins in an axon than does contact with a cell presenting other adhesion proteins and the full-length NLG-1 (Fig. 1A–C). We have also shown that synaptic vesicles and active zone proteins can appear within tens of minutes of contact (Fig. 2) and that, within 1 h, the site has activity-dependent vesicle cycling that is characteristic of transmitter release (Fig. 1C). In contrast, the induction is delayed when full-length NLG-1 is the trigger for

differentiation (Fig. 1D,E). This led us to extend previous studies on soluble ectodomains, and much longer (24–48 h) interaction times between the full-length proteins presented on the surfaces of contacting cells, to show that NLG-1 Δ B is able to concentrate more α -NRX in HEK293 cells (Fig. 3) and in neurons (Fig. 4). In addition, time-lapse experiment in neurons validated that NLG-1 Δ B shows no preference between α -NRX and β -NRX (Fig. 5), whereas NLG-1 prefers β -NRX over α -NRX. We further found that an associated scaffolding protein, CASK, an intracellular partner for both α - and β -NRXs (Hata et al., 1996), accumulates equally fast at NLG-1 and NLG-1 Δ B contacts (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Knockdown of α -Neurexin-1, 2, and 3 delays Neuroligin-1 Δ B-induced synapse formation

Our above results suggest that NLG-1 Δ B is uniquely capable of inducing the fast formation of presynaptic nerve terminals because of its interaction with α -NRXs. If this is correct, then we would expect that a reduction of expression levels of α -NRX in the neuron would delay this process. To test this, we used RNA interference to knock down α -NRX protein levels in cultural neurons. Rats have three α -NRXs, all of which are known to bind NLG-1 Δ B (Boucard et al., 2005) and found in the hippocampus (Püschel and Betz, 1995). Four shRNA sequences were designed to knock down expression of these three α -NRXs by targeting unique α -NRXs sequences not present in the β form (see Materials and Methods). Each shRNA was driven by its own H1 RNA promoter, and the four were incorporated in tandem into a single pSuper–GFP vector (Stove et al., 2006) to ensure that the four α -NRXs shRNAs would be transcribed in the same cell. Coexpression of this quadruple shRNA vector in HEK293 cells along with each of the FLAG– α -NRX-1, -2, or -3 resulted in a strong reduction in expression but had no effect on NLG-1 Δ B (Fig. 6A). Having demonstrated the efficacy of the shRNA vector, we next transfected it into neurons by Nucleofection, achieving a transfection rate of 10–30%, based on GFP expression. HEK293 cells coexpressing NLG-1 Δ B and mRFP were cocultured with the shRNA-transfected neurons, and the cultures were stained for endogenous BSN at 1, 3, and 6 h after HEK293–neuron contact. We found that α -NRX knockdown reduced the number of synapses induced by NLG-1 Δ B on transfected neurons at both 3 and 6 h after contact, indicating that the rapid synapse formation induced by NLG-1 Δ B was delayed (Fig. 6B,C). This result supports the interpretation that

α -NRX is a key mediator of rapid synapse induction by NLG-1 splice variants.

The glycosylation site in insert B reduces affinity for α -Neurexin and triggers a slow synapse formation

If the affinity of NLG-1ΔB for α -NRX makes it uniquely capable of inducing fast synapse formation, then we would predict that the manipulation of the affinity of NLG-1 for α -NRX should change the rate of synapse induction by NLG-1. Insert B of NLG-1 is known to lower its affinity for the soluble domain of α -NRX as a result of an N-linked glycosylation site (N303) located within insert B (Boucard et al., 2005). We wondered whether mutation of this site to serine (NLG-1-N303S) would increase NLG-1 affinity to α -NRX in cells. To test this, we performed an HEK293 cell binding assay with pDisplay-YFP as a membrane marker. We found that the relative density of α -NRX at the junction with NLG-1-N303S-expressing cells was 1.84 ± 0.22 , similar to what was seen at junctions with cells expressing NLG-1ΔB (2.09 ± 0.22) and significantly higher than what was seen at contacts with NLG-1-expressing cells (1.20 ± 0.08) (Fig. 3A, $n = 10$ for each group). In contrast, the recruitment of β -NRX was the same for NLG-1, NLG-1ΔB, and NLG-1-N303S (Fig. 3A, $n = 10$ for each group).

Having observed that the apparent affinity of NLG-1-N303S for α -NRX was similar to that of NLG-1ΔB, we asked whether NLG-1-N303S would also induce new pre-synaptic terminals with the greater speed of NLG-1ΔB. We found that, within 1 h of contact, native Bassoon accumulated significantly around HEK293 cells transfected with NLG-1-N303S (NLG-1, $1.2 \pm 0.7\%$; NLG-1-N303S, $10.4 \pm 3.8\%$; $n = 6$ and 10 respectively; $p < 0.05$, Mann-Whitney rank-sum test), as found with NLG-1ΔB but not NLG-1 (Fig. 1). This finding supports the idea that the accelerated rate of synapse induction correlates with the recruitment of α -NRX in the axon at the site of contact with the NLG-1-presenting cell.

Intracellular clustering of NLG-1 by PSD-95 accelerates Bassoon recruitment

If the affinity of NLG-1 for α -NRX determined the rate with which it induces presynaptic differentiation, as proposed above, then clustering of NLG-1 in the membrane would be predicted to increase binding and accelerate presynaptic differentiation. NLG-1 forms oligomers on its own (Scheiffele et al., 2000; Comoletti et al., 2007), but it also binds the multimerizing scaffolding protein PSD-95 (Hsueh et al., 1997; Hsueh and Sheng, 1999), suggesting that PSD-95 might increase NLG-1 clustering and, consequently, increase α -NRX binding and favor synapse induction. We tested this notion by comparing the recruitment of active zone pro-

Figure 5. Time lapse of axonal NRX recruitment to NLG-1ΔB contacts. **A**, Time-lapse imaging shows recruitment to a new site of contact with an NLG-1ΔB-expressing HEK293 cell of α -NRX-mChFP and β -NRX-mYFP in same axon of a doubly transfected neuron. Left shows images in cyan and yellow channels taken before contact, 1 and 2 min after contact, and every 2 min thereafter for 1 h. HEK293 cell is marked by white circle. Right displays the line-scan display of fluorescence along axon (between yellow arrowheads in images on left). **B**, The ratio of α -NRX to β -NRX accumulation at 1 h after contact ($n = 5$ for each group).

teins in hippocampal axons at contacts with HEK293 cells that expressed either NLG-1 alone or NLG-1 along with PSD-95-mRFP. Adding PSD-95-mRFP was found to increase the recruitment of Bassoon (Fig. 7). Coexpression of PSD-95-mRFP also boosted Bassoon recruitment to NLG-1ΔB contacts (Fig. 7), indicating that Bassoon recruitment by NLG-1ΔB can be further enhanced.

Neuroligin-1ΔB exhibits an enhanced ability to increase synaptic density in neurons

Overexpression of NLG-1 in neurons is known to increase synaptic density (Dean et al., 2003; Boucard et al., 2005; Sara et al.,

Figure 6. α-NRX-1, -2, -3 triple knockdown reduces the number of synapses induced by NLG-1ΔB. **A**, Western blot of HEK293 cells cotransfected with FLAG-tagged α-NRX-1, α-NRX-2, α-NRX-3, or NLG-1ΔB in combination with either the quadruple α-NRX shRNA vector or the pSuper control. The cells were lysed and analyzed 2 d after transfection. The expression of α-NRX proteins was substantially reduced by coexpression of the shRNA vector compared with the pSuper control, indicating that the quadruple shRNA effectively knocks down α-NRX expression. The α-NRX shRNA vector has no effect on the expression of NLG-1ΔB, indicating that its suppression of α-NRXs is specific. **B**, Neurons nucleofected immediately after dissection with the quadruple α-NRX shRNA vector, or the pSuper control, were cocultured at DIV 5 with NLG-1ΔB-expressing HEK293 cells for different amounts of time and then stained with antibodies against BSN as the marker for synapses. The quadruple α-NRX shRNA vector reduced the number of synapses at the sites of contact with the NLG-1ΔB-expressing HEK293 cell at 3 and 6 h after contact ($n = 74, 45$, and 44 neuron–HEK293 contacts, respectively, for pSuper control at 1, 3, and 6 h; $n = 57, 43$, and 35 neuron–HEK293 contacts, respectively, for the quadruple α-NRX shRNA vector at 1, 3, and 6 h). $*p < 0.05$, Student's *t* test. **C**, Examples of pSuper or quadruple α-NRX shRNA vector transfected neurons (green) in contact with NLG-1ΔB-expressing HEK293 cells (blue) 3 h after HEK293–neuron contact. Bassoon-stained synapses (red) between transfected neurons and HEK293 cells were fewer in number for those neurons transfected with the α-NRX shRNA vector compared with those with pSuper control.

2005; Ko et al., 2009b). We asked whether overexpression of NLG-1ΔB in neurons, with its ability to accelerate presynaptic differentiation, would further increase the density of synapses at neuro-neuronal contacts at later time points. DIV 9 neurons were grown for 16 h after transfection with CD8–YFP alone, as a control, or CD8–YFP along with either NLG-1ΔB or NLG-1. The cultures were then stained with anti-BSN antibodies (Fig. 8A). Because the density of synapses on a CD8–YFP-transfected neuron was highly correlated with the average synaptic density within the field of view ($R^2 = 0.77$) (Fig. 8B), we evaluated the data with ANCOVA by fitting the data to a linear additive parallel lines model, in which

Figure 7. PSD-95 coexpression with NLG in HEK293 cell accelerates recruitment of presynaptic machinery to contact site with axon. **A**, Representative examples of increase in the recruitment of endogenous BSN (green) by NLG-1 or NLG-1ΔB because of coexpression with PSD-95–mRFP (red) at 1 h after contact. **B**, Quantification of **A**. NLG-1ΔB with PSD-95–mRFP induced the highest amount of BSN recruitment, whereas NLG-1ΔB alone, and NLG-1 with PSD-95, induced less, but still significantly higher than NLG-1 alone (Kruskal–Wallis one-way ANOVA, followed by multiple comparisons, $n = 10$ for each group, $*p < 0.05$). ΔB, NLG-1ΔB; 1, full-length NLG-1. Scale bar, 10 μm.

the slope is taken to be an invariant function of the number of processes (as gauged by the number of synapses) in the field of view and greater potency of synapse induction by the adhesion protein results in higher values along the *y*-axis (see Materials and Methods). We found that, on average, NLG-1ΔB increased Bassoon puncta density over the CD8–YFP control by 1.2 ± 0.42 synapses/10 μm length of dendrite, whereas NLG-1 increased Bassoon puncta density by only half that amount, 0.6 ± 0.44 synapses/10 μm (Fig. 8C). Thus, not only is NLG-1ΔB more potent in inducing the recruitment of presynaptic machinery than full-length NLG-1 at contacts with surrogate postsynaptic cells, but it is also more potent at axo-dendritic contacts.

Discussion

A number of postsynaptic adhesion molecules have been implicated in synapse formation. We examined several of these and asked which is able to induce presynaptic differentiation at a rate comparable with the one observed at new neuro-neuronal contacts (Ahmari et al., 2000; Friedman et al., 2000; Bresler et al., 2004; Garner et al., 2006). We found that, between the full-length NLG-1 and its alternative splice product NLG-1ΔB, SynCAM, EphB2, NGL-2, and N-Cadherin, only NLG-1ΔB induces fast presynaptic differentiation (Fig. 1A,B).

The unique ability of NLG-1ΔB to rapidly induce functional presynaptic terminals was mimicked by removing the N-linked glycosylation site (N303) within insert B from the full-length NLG-1. As shown previously in isolated ectodomains mixed in solution (Boucard et al., 2005), we found that absence of insert B or its glycosylation site makes NLG-1 bind more strongly to α-NRX (Fig. 3A). The results suggest that the fast presynaptic differentiation results from the recruitment of α-NRX to the site

of contact with the NLG-presenting cell. Indeed, mutants of NLG-1 that are unable to bind α -NRXs have been shown previously to fail in synapse induction (Ko et al., 2009b), and LRRTM2, which binds both α - and β -NRX, was found to be more potent than full-length NLG-1 (Siddiqui et al., 2010). Consistent with this, we found that NLG-1 Δ B recruits more α -NRX than does the full-length NLG-1 in HEK293 cells (Fig. 3) and neurons (Fig. 4). Moreover, we found that knockdown of all three α -NRXs delays the rate of synapse formation induced by NLG-1 Δ B (Fig. 6), demonstrating that NLG-1 Δ B- α -NRX interaction is vital to rapid presynaptic differentiation.

The knockdown of α -NRXs results in fewer induced synapses at 3 and 6 h after contact (Fig. 6B). No difference was observed at 1 h, but this likely reflects the fact that the biggest divergence between presynaptic differentiation induced by NLG-1 and NLG-1 Δ B occurs between 2 and 12 h (Fig. 1D), whereas the difference at 1 h is small and close to the detection limit (Fig. 1B,D).

Although affinity chromatography on soluble ectodomains suggests that NLG-1 exclusively interacts with β -NRX (Ichtkhenko et al., 1995; Boucard et al., 2005), we found that full-length NLG-1 presented on a contacting cell is capable of recruiting α -NRX. This may explain why, although delayed compared with NLG-1 Δ B, NLG-1 eventually induces presynaptic terminals capable of activity-dependent vesicle release. It is possible that clustering in the membrane and/or high expression in HEK293 cells partially overcomes the relatively low affinity of NLG-1 and recruits sufficient α -NRX to induce new synapses. Consistent with this, we find that PSD-95, a postsynaptic scaffolding protein that clusters postsynaptic proteins (including NLG-1) (Hsueh et al., 1997; Hsueh and Sheng, 1999), accelerates the presynaptic differentiation triggered by NLG-1. In a similar vein, it was suggested recently that glial factor Thrombospondin-1 accelerates presynaptic differentiation in neurons by clustering NLG-1 (Xu et al., 2010).

Overexpression of NLG-1 in neurons was shown previously to increase the density of synapses in hippocampal cultures, as assayed by staining for presynaptic proteins (Dean et al., 2003; Boucard et al., 2005; Sara et al., 2005; Ko et al., 2009b). We found that overexpression of NLG-1 Δ B increases synaptic density even more. Although this is in contrast to previous reports (Boucard et al., 2005; Ko et al., 2009b), which showed that NLG- α -NRX interaction influences synaptic size rather than density, two possible explanations may account for the discrepancy. First, in our study, the fluorescent protein tag was inserted into the extracellular domain of NLG-1, whereas in the previous studies (Boucard et al., 2005; Ko et al., 2009b), it was inserted into the intracellular domain, in which it might influence protein trafficking or signaling. Second, our analysis took into account the synaptic density within the field of view. All in all, our results support the idea that, in addition to the role of NLG- α -NRX interaction in the determi-

Figure 8. Overexpression of NLG-1 or NLG-1 Δ B in neurons elevates synaptic density to different levels. **A**, Representative dendrites from neurons transfected with CD8-YFP, CD8-YFP + NLG-1, or CD8-YFP + NLG-1 Δ B. Antibody staining for native presynaptic BSN (red) identifies all presynaptic nerve terminals, and CD8-YFP (green) defines the dendrites of the NLG-transfected neurons. **B**, Synaptic density (BSN puncta per 10 μ m dendrite length of transfected neuron) plotted against total number of synapses (BSN puncta in each field of view). The two variables are strongly correlated ($R^2 = 0.77$) when fitted with three parallel lines: CD8-YFP, green; NLG-1, blue; NLG-1 Δ B, red. For detailed analysis and comparisons with other models, see Materials and Methods. In brief, parallel-line model best described the data with the lowest number ($= 4$) of parameters. Separate regression of each group does not decrease residuals significantly with additional parameters, whereas other models with the same or fewer parameters increase residuals. **C**, The result of ANCOVA using a linear additive parallel lines model. The synaptic density per 10 μ m of each group when the total number of synapses in a field of view is 1000 is shown with the SEs of their estimates (CD8, 1.97 ± 0.16 ; NLG-1, 2.58 ± 0.25 ; NLG-1 Δ B, 3.18 ± 0.24 synapses/10 μ m dendrite; $n = 40$ –50 neurons per condition). The analysis indicates that both NLG-1- and NLG-1 Δ B-expressing dendrites have significantly elevated synaptic densities compared with CD8-YFP alone and that NLG-1 Δ B-expressing dendrites have the highest ($*p < 0.05$). The experiment was repeated once, and the same conclusion was obtained (when the total number of synapses in a field of view is 1000, the synaptic densities are as follows: CD8, 1.86 ± 0.11 synapses/10 μ m dendrite; NLG-1, 2.38 ± 0.10 synapses/10 μ m dendrite; NLG-1 Δ B, 2.62 ± 0.10 synapses/10 μ m dendrite; $n = 39$ –40 neurons per condition).

nation of excitatory versus inhibitory synaptic connections (Chih et al., 2005), this interaction is a key to rapid synapse formation.

With fluorescent imaging and immunocytochemistry over 24 h, we were able to define the time course of presynaptic differentiation induced by NLG-1 and NLG-1 Δ B. The initial adhesion between α -NRX and NLG-1, or its splice variants, occurs quickly: within a few minutes (Fig. 5A), reaching a maximum within 1 h (Fig. 4B), consistent with a role in triggering synapse formation. Downstream components such as active zone proteins and synaptic vesicles (marked by BSN and SYN, respectively) arrive at the new contact sites within tens of minutes to hours, depending on the level of α -NRX (Figs. 1D, 4D). Interestingly, at the site of contact with the NLG-expressing HEK293 cells, there is a high degree of colocalization of active zone proteins (including Bassoon) and synaptic vesicles (containing Synapsin), and this was seen at all time points after contact (Fig. 1E), indicating that their recruitments are coordinated.

Although the amount of α -NRX recruitment and the rate of formation of new synapses are greater for NLG-1 Δ B, the number of NLG-1-triggered synapses catches up after a few hours when the postsynaptic element is an HEK293 cell. This suggests that the assembly of presynaptic components is rate limiting, but once presynaptic assembly has occurred the synapses are stable. Al-

though the HEK293 cell is a powerful minimal model for the dendrite, permitting us to define the molecular interactions and the time and place of contact with the axon, however, it lacks the dynamic nature of the morphology of the dendrite. In the natural case of axo-dendritic contacts, in which the presentation of the postsynaptic adhesion protein is made by the dynamic dendritic filopodia, the pre-post contact is transient and, as a consequence, the speed of the presynaptic adhesion complex formation and the release machinery recruitment will likely make a significant difference to the number of synapses that form. This would be expected to favor NLG-1 Δ B for its more efficient recruitment of α -NRX. Indeed, overexpression of NLG-1 Δ B results in a larger number of neuro-neuronal synapses than does NLG-1 (Fig. 8C).

An important objective for future studies will be to determine why α -NRX induces quicker presynaptic differentiation. α -NRX and β -NRX have a common cytoplasmic domain but differ in their extracellular domains. Because the recruitment of β -NRX is insufficient for fast maturation, acceleration of synapse formation appears likely to require the part of the protein that is unique to α -NRX, namely the extracellular domain that only exists in α -NRX (Ushkaryov et al., 1992; Ushkaryov and Südhof, 1993). This raises the possibility that extracellular α -NRX binding partners may be involved in rapid synapse induction. These include Neurexophilin (Petrenko et al., 1996) and dystroglycans (Sugita et al., 2001). However, Neurexophilin-1 is only found in a subset of inhibitory neurons (Petrenko et al., 1996), Neurexophilin-3 is absent from hippocampus (Beglopoulos et al., 2005), and dystroglycans are only found at inhibitory synapses (Lévi et al., 2002), making them unlikely candidates to participate in accelerating synapse formation at all synapses. In addition, although synaptic N-type Ca^{2+} channels are significantly reduced in α -NRX triple knock-out mice, they do not interact with α -NRX directly (Missler et al., 2003), suggesting that there may well be other, as yet unidentified, binding partners.

In conclusion, although multiple synaptogenic adhesion molecules are able to induce presynaptic terminal differentiation, we showed that the induction rates by several of these molecules is much slower than what is seen during the formation of synapses at new sites of axo-dendritic contact. The exceptions were an alternative splice product of NLG-1 that was missing its insert B and a mutant of NLG-1 missing just the N-linked glycosylation site that is situated within insert B. Using a combination of immunostaining and time-lapse microscopy, we found that the fast presynaptic induction by the NLG-1 Δ B alternative splice product is associated with its stronger affinity to α -NRXs. This provides a potential mechanism for neurons to regulate their synapse formation rates during development to alter the connectivity of the network.

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