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A Splice Code for *trans*-Synaptic Cell Adhesion Mediated by Binding of Neuroligin 1 to α- and β-Neurexins

Report

Antony A. Boucard,[1] Alexander A. Chubykin,[1] Davide Comoletti,[4] Palmer Taylor,[4] and Thomas C. Südhof[1,2,3,*]
[1]Center for Basic Neuroscience
[2]Department of Molecular Genetics
[3]Howard Hughes Medical Institute
The University of Texas Southwestern Medical Center
6000 Harry Hines Boulevard NA4.118
Dallas, Texas 75390
[4]Department of Pharmacology
University of California, San Diego
9500 Gilman Drive
La Jolla, California 92093

Summary

Previous studies suggested that postsynaptic neuroligins form a *trans*-synaptic complex with presynaptic β-neurexins, but not with presynaptic α-neurexins. Unexpectedly, we now find that neuroligins also bind α-neurexins and that α- and β-neurexin binding by neuroligin 1 is regulated by alternative splicing of neuroligin 1 (at splice site B) and of neurexins (at splice site 4). In neuroligin 1, splice site B is a master switch that determines α-neurexin binding but leaves β-neurexin binding largely unaffected, whereas alternative splicing of neurexins modulates neuroligin binding. Moreover, neuroligin 1 splice variants with distinct neurexin binding properties differentially regulate synaptogenesis: neuroligin 1 that binds only β-neurexins potently stimulates synapse formation, whereas neuroligin 1 that binds to both α- and β-neurexins more effectively promotes synapse expansion. These findings suggest that neuroligin binding to α- and β-neurexins mediates *trans*-synaptic cell adhesion but has distinct effects on synapse formation, indicating that expression of different neuroligin and neurexin isoforms specifies a *trans*-synaptic signaling code.

Introduction

Neurexins and neuroligins are pre- and postsynaptic cell surface proteins, respectively, that may function as *trans*-synaptic cell adhesion molecules (Ushkaryov et al., 1992; Ichtchenko et al., 1995, 1996; Song et al., 1999; Varoqueaux et al., 2004; reviewed in Yamagata et al., 2003). Vertebrates contain three large neurexin genes, each of which expresses longer α- and shorter β-neurexins, producing six principal neurexins (neurexins 1α, 2α, 3α and 1β, 2β, 3β; Ushkaryov et al., 1992, 1994; Rowen et al., 2002; Tabuchi and Südhof, 2002; Figure 1A). These six principal neurexins are diversified by extensive alternative splicing at five conserved sites (referred to as splice sites 1 to 5), resulting in up to 1000 variants (Ullrich et al., 1995). Neuroligins were identified based on their binding to β-neurexins and differ from neurexins in that they have a relatively simple structure and are only differentially spliced at two positions (splice sites A and B; Figure 2A; Ichtchenko et al., 1995, 1996).

Neuroligin 1 was found to bind tightly only to β-neurexins that lack an insert in splice site 4 (Ichtchenko et al., 1995, 1996). In neurexins, splice site 4 is expressed in variants containing or lacking a 30 residue insert and is developmentally and spatially regulated (Ullrich et al., 1995). The synaptic localization and interaction of neuroligins and neurexins, including their ability to mediate cell adhesion (Nguyen and Südhof, 1997), prompted the hypothesis that postsynaptic neuroligins form a *trans*-synaptic complex with presynaptic β-neurexins (Ichtchenko et al., 1995). This hypothesis was confirmed by the observation that neuroligin 1, when expressed in nonneuronal HEK293 cells, induces presynaptic differentiation in cocultured pontine explants or hippocampal neurons (Scheiffele et al., 2000; Fu et al., 2003). Similarly, β-neurexins, when expressed in nonneuronal cells, induce postsynaptic specializations in cocultured neurons (Graf et al., 2004). Moreover, RNA interference studies indicated that "knockdowns" of neuroligins may hinder synapse formation (Chih et al., 2005). Since neuroligins constitutively dimerize via their extracellular domain (Comoletti et al., 2003), they may induce presynaptic differentiation by clustering β-neurexins (Dean et al., 2003). The importance of the neuroligin/neurexin complex for normal brain function is highlighted by the finding that, in humans, mutations in X-chromosomal neuroligin genes are associated with autistic spectrum disorders and/or mental retardation (Jamain et al., 2003; Yan et al., 2004).

The overexpression and RNA interference experiments suggested a function for β-neurexins and neuroligins in synapse formation but provided little information about α-neurexins. Knockout (KO) studies revealed that deletion of α-neurexins was perinatally lethal because synaptic function was severely impaired without a major defect in synapse formation (Missler et al., 2003; Zhang et al., 2005; Kattenstroth et al., 2004). The α-neurexin KO findings established a role for α-neurexins in mature synapses that is distinct from the function suggested for β-neurexins in overexpression studies (Dean et al., 2003; Graf et al., 2004). However, α- and β-neurexins are largely composed of the same sequences (Figure 1A), which would be more consistent with similar instead of radically different functions. Although further genetic and overexpression studies likely will help to clarify this conundrum, a major impediment to understanding the function of neurexins is that little is known about extracellular α-neurexin ligands. At present, the only identified ligands for α-neurexins are dystroglycan (Sugita et al., 2001) and neurexophilins (Missler et al., 1998a). Dystroglycan, a ubiquitous cell adhesion molecule, performs an essential function in neurons (Moore et al., 2002) that, however, does not appear to involve synapse formation (Graf et al., 2004). Neurexophilins are secreted proteins resembling neuropeptides that may activate α-neurexin signaling but are not involved in *trans*-synaptic cell adhesion (Missler et al., 1998b).

*Correspondence: thomas.sudhof@utsouthwestern.edu

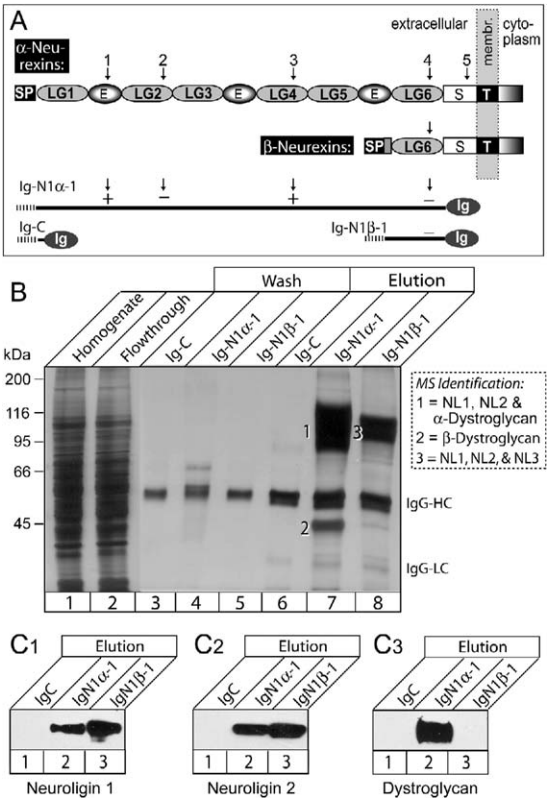

Figure 1. Affinity Purification of Neuroligins on Immobilized Neurexin 1α

(A) Domain structures of α- and β-neurexins (top), and composition of recombinant Ig fusion proteins used for affinity purifications (bottom). Abbreviations: SP, signal peptide; LG1 to LG6, laminin G domains 1 to 6 (also called LNS domains for laminin/neurexin/sex hormone binding globulin domains); E, EGF-like sequences; S, carbohydrate attachment site; T, transmembrane region. Numbered arrows identify positions of canonical sites of alternative splicing; in the Ig fusion proteins, + and − signs identify the presence or absence of inserts in the corresponding sites of alternative splicing.

(B) Affinity chromatography experiments. Solubilized proteins from total rat brain homogenate (lane 1) were applied to a column containing immobilized Ig control (Ig-C), Ig-N1α-1, and Ig-N1β-1, and the flowthrough was collected (lane 2). The column was washed extensively with loading buffer (lanes 3–5) and eluted with 0.5 M NaCl (data not shown) and 1.0 M NaCl (lanes 6–8). Fractions were analyzed by SDS-PAGE and silver staining. The major specific bands (labeled 1–3 on the gel) were identified by mass spectroscopy as indicated on the right ("MS Identification").

(C) Immunoblotting analysis of proteins bound to Ig-C, Ig-N1α-1, and Ig-N1β-1 and eluted in high salt.

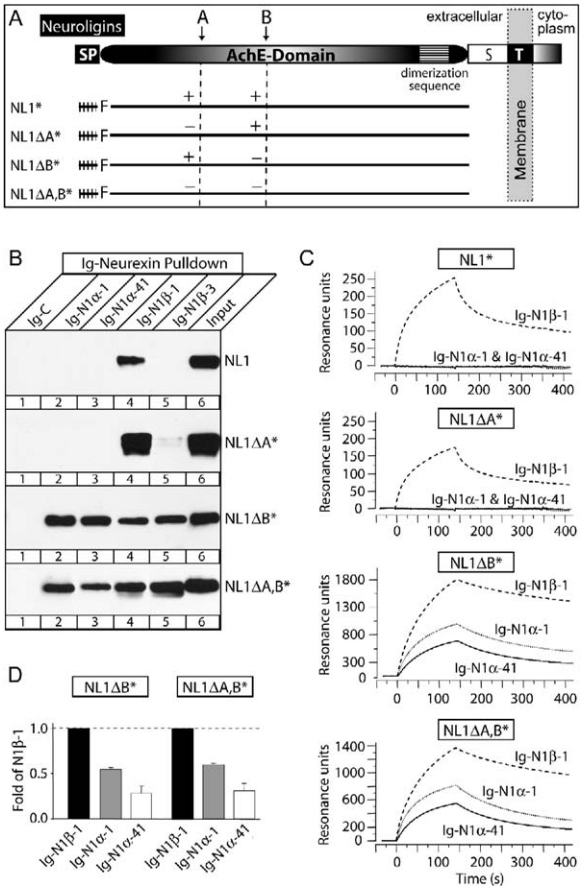

Figure 2. Regulation of Neurexin Binding by Alternative Splicing of Neuroligin 1

(A) Domain structure of neuroligins (top), and composition of recombinant Flag-tagged neuroligins used as ligands for neurexins (bottom). Abbreviations: SP, signal peptide; AchE-domain, acetylcholinesterase-like domain including the dimerization sequence; S, O-linked carbohydrate attachment site; T, transmembrane region; arrows labeled "A" and "B" identify positions of canonical alternative splice sites, with + and − signs signifying the presence or absence of an insert in the corresponding site.

(B) Pull-down of recombinant neuroligin 1 variants by immobilized Ig neurexin fusion proteins. Secreted neuroligins from the medium of transfected COS cells were bound to the neurexin Ig fusion proteins indicated on top of the diagram (Ig-N1α-1 and -41 and Ig-N1β-1 and -3 contain the full-length extracellular sequences of neurexins 1α and 1β without and with an insert in splice site 4, respectively; see Figure 4A). Bound neuroligins were visualized by immunoblotting with an NL1-specific antibody.

(C) Binding analysis using surface plasmon resonance. Indicated neuroligin 1 splice variants (111 nM) were injected over Ig neurexin surfaces for 150 s, followed by buffer alone. Three curves generated by the same NL1 injection over surfaces containing the three different neurexins were overlaid in each panel.

(D) Comparison of the apparent neuroligin 1 affinities of neurexin 1β with neurexin 1α containing (Ig-N1α-41) or lacking (Ig-N1α-1) an insert in splice site 4 (n = 3 injections; RU, resonance unit). Error bars represent means ± SDs. Note that only neuroligin 1 variants lacking an insert in splice site B were tested because, with an insert in splice site B, no neuroligin 1 binding to neurexin 1α was detected.

To address this conundrum, we searched for α-neurexin ligands that might be involved in *trans*-synaptic cell adhesion. Surprisingly, we identified neuroligins as major interaction partners of α-neurexins. We found that a splice variant of neuroligin 1 that lacks an insert in splice site B and that was not previously tested (Ichtchenko et al., 1995) avidly interacts with α-neurexins, as do neuroligins 2 and 3. Neuronal expression of a neuroligin 1 variant that only binds to β-neurexins promotes synapse formation more potently, but synapse expansion less effectively, than a neuroligin 1 variant that binds to both α- and β-neurexins. Our data thus suggest that α- and β-neurexins may execute a shared function in *trans*-synaptic cell adhesion by binding to neuroligins, with α-neurexins performing additional roles via interactions that are not mediated by β-neurexins and that may explain the α-neurexin KO phenotype (Missler et al., 2003).

Results

We chose a biochemical approach to search for endogenous ligands of α-neurexins. We produced Ig fusion proteins that include almost the entire extracellular domains of neurexin 1α (Ig-N1α-1) or neurexin 1β (Ig-N1β-1), and an Ig control protein (Ig-C) that contains only the 18 N-terminal residues of mature neurexin 1α (Figure 1A). We then performed affinity chromatography experiments with rat brain proteins on the Ig fusion proteins that were immobilized on protein A-Sepharose and analyzed the eluents on silver-stained SDS polyacrylamide gels (Figure 1B). A heterogeneous group of proteins of ~90–120 kDa and a single protein of ~50 kDa were purified on neurexin 1α, while only the ~90–120 kDa proteins were bound to neurexin 1β. Mass spectroscopy showed that the ~90–120 kDa proteins purified on neurexin 1α included neuroligins 1 and 2 and α-dystroglycan, and the ~50 kDa band was composed of β-dystroglycan, while the ~90–120 kDa proteins purified on neurexin 1β contained neuroligins 1, 2, and 3 but not α-dystroglycan. No significant binding of a protein to the Ig control column was detected, except for IgG heavy and light chains (IgG-HC and IgG-LC) that were present in all eluents, presumably because endogenous IgG in the brain homogenates bound to protein A-Sepharose (Figure 1B). No other proteins with extracellular sequences were identified in the neurexin affinity-purified fractions, suggesting that only neuroligins and dystroglycan tightly interact with neurexin 1α, and only neuroligins tightly bind to neurexin 1β.

Immunoblotting confirmed specific binding of brain neuroligins 1 and 2 to both neurexin 1α and neurexin 1β, whereas dystroglycan only bound to neurexin 1α (Figure 1C), and control membrane proteins such as SynCAM did not bind to any neurexin (data not shown). Moreover, binding of full-length recombinant neuroligins 2 and 3 to immobilized neurexin 1α further corroborated general binding of neuroligins to α-neurexins (Figure S1 in the Supplemental Data available with this article online). Finally, we also tested whether endogenous α-neurexins in brain can be affinity purified on immobilized recombinant neuroligin 1. In the neuroligin 1 eluent, we observed a single major protein of ~175 kDa that was absent from control eluent (Figure S2), and was found by sequencing to contain a mixture of neurexins 1α, 2α, and 3α. In conjunction with the experiments of Figure 1 and Figure S1, these results indicate that α-neurexins generally interact with neuroligins.

Conceptually, the idea that neuroligins bind to α-neurexins is attractive because binding of neuroligins to both α- and β-neurexins would suggest a general function of all neurexins in neuroligin-dependent *trans*-synaptic cell adhesion. However, different from the present affinity chromatography experiments, we previously failed to uncover binding of α-neurexins to neuroligin 1 (Ichtchenko et al., 1995). To address this discrepancy, we produced recombinant neuroligin 1 proteins that were tagged with an N-terminal Flag epitope and were truncated after the dimerization sequence at the C terminus of the acetylcholinesterase-like domain (Figure 2A). Since neuroligin 1 possesses two alternatively spliced sequences referred to as splice sites A and B (Ichtchenko et al., 1995), we tested the binding of all four splice combinations of neuroligin 1 to immobilized neurexin Ig fusion proteins. In these experiments, we examined neurexin 1α and 1β variants that either included (Ig-N1α-41 and Ig-N1β-3) or lacked an insert in splice site 4 (Ig-N1α-1 and Ig-N1β-1), and used Ig-C as a control. We observed that neuroligin 1 containing an insert in splice site B—with or without an insert in splice site A—exclusively bound to neurexin 1β lacking an insert in splice site 4. In contrast, neuroligin 1 lacking an insert in splice site B—again with or without an insert in splice site A—bound to both neurexin 1α and 1β, and to both of the splice variants tested for either neurexin (Figure 2B).

These results suggest that alternative splicing of neuroligin 1 at splice site B (which involves insertion or deletion of eight residues that are completely conserved in mammals) regulates its binding to neurexins. To confirm this possibility, we immobilized purified neuroligins on agarose beads containing anti-Flag antibodies and examined the binding of soluble Ig neurexins (Figure S3). Bound Ig proteins were probed with anti-human IgG antibody. The results of the neuroligin pull-downs replicated those of the neurexin pull-downs with one exception: the neuroligin pull-downs detected weak binding of insert-positive β-neurexin to immobilized splice site B-containing neuroligin 1 (Figure S3), whereas the neurexin pull-downs did not (Figure 2B). This result indicates that insert-positive neuroligin 1 exhibits a low but finite affinity for insert-positive neurexin 1β.

To quantify better the information obtained with classical pull-down assays, we monitored binding between neuroligin 1 and the neurexin 1α with or without splice insert 4 by surface plasmon resonance (SPR). For complete comparisons, equimolar amount of Ig-N1β-1, Ig-N1α-1, and Ig-N1α-41 were coupled to a CM5 microchip, and the same concentration of the four neuroligin 1 splice variants was injected. As suggested by the pull-downs, only neuroligin 1 lacking the splice site B insert (irrespective of the presence of splice insert A) bound to both splice variants of Ig-N1α, indicating an absolute inhibitory function of splice insert B (Figure 2C). Since measurements of association are made in real time, affinities can be estimated by comparing the maximum binding of the same nonsaturating concentration in each condition. This comparison demonstrated that the absolute affinity of neurexin 1α for neuroligin 1 lacking splice site B is ~2-fold lower than that of neurexin 1β and is additionally regulated (again ~2-fold) by alternative splicing of neurexin 1α at splice site 4 (Figure 2D).

Finally, to further confirm the affinity chromatography and SPR conclusions by yet another assay, we expressed all four splice site combinations of full-length Flag-tagged neuroligin 1 in HEK293 cells. We then bound recombinant Ig fusion proteins of neurexins to the transfected cells, using Ig-C as a control. We fixed the cells without detergent permeabilization and stained them by immunofluorescence for the Ig fusion proteins (to measure binding of the Ig fusion proteins) and for neuroligin 1 (to ensure that the various neuroligins were expressed on the cell surface). We found that neurexin 1β, irrespective of whether splice site 4 contained or lacked an insert, specifically bound to all neuroligin 1 splice variants. In contrast, neurexin 1α variants only

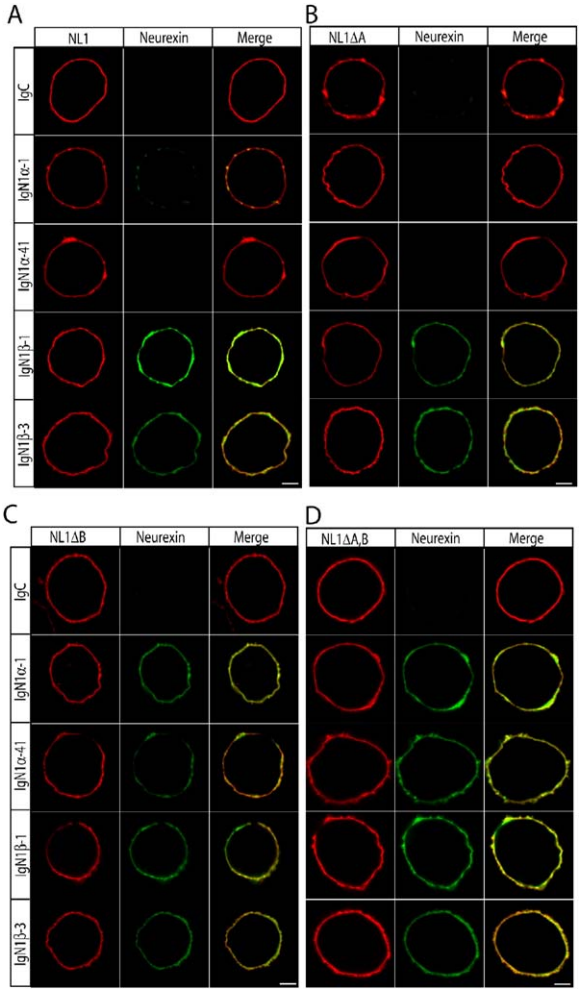

Figure 3. Binding of Ig Neurexins to Neuroligin 1 Splice Variants Expressed on the Surface of HEK293 Cells

Full-length neuroligin 1 either containing an insert in both splice site A and splice site B (NL1; [A]), lacking an insert in either splice site A (NL1ΔA; [B]) or B (NL1ΔB; [C]), or lacking an insert in both splice site A and splice site B (NL1ΔA,B; [D]) were expressed in transfected HEK293 cells. Transfected cells were fixed in the absence of detergent and incubated with 0.15 μM Ig neurexins or Ig control proteins. Cell surface proteins were visualized with antibodies to the Flag epitope (red) or to human Ig (green). In all panels, the right images show the merged picture of the two labeling reactions, with coincident labeling shown in yellow.

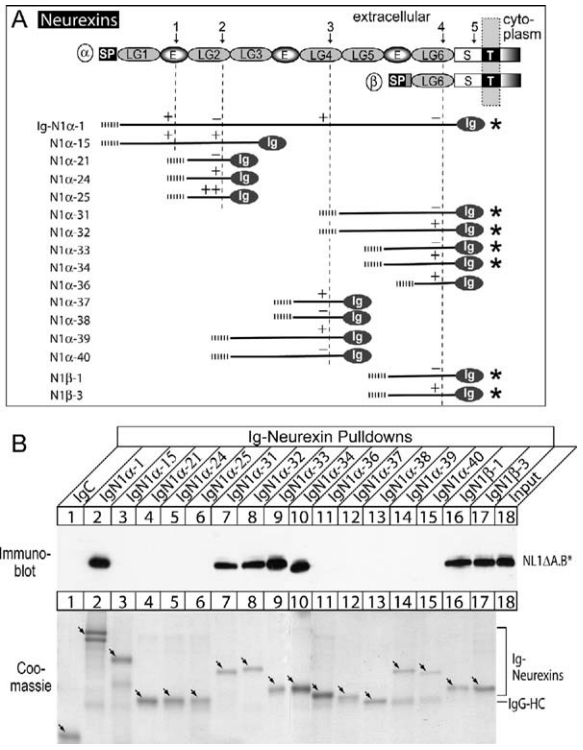

Figure 4. Binding Site in Neurexin 1α for Neuroligin 1

(A) Domain structures of α- and β-neurexins, and structures of neurexin Ig fusion proteins used for mapping the neuroligin binding site on neurexins. Please see the legend to Figure 1A for an explanation of the abbreviations used.

(B) Equivalent molar amounts of the immobilized Ig neurexin fusion proteins were incubated with 0.1 μg of NL1ΔA,B* (truncated secreted neuroligin 1 lacking inserts in splice sites A and B; see Figure 2A) in the presence of Ca^{2+}. Bound proteins were analyzed by SDS-PAGE and immunoblotting for neuroligin 1 (top), and Ig proteins were assessed by Coomassie blue staining (bottom).

bound to neuroligin 1 lacking an insert in splice site B. No binding of Ig control protein to any neuroligin 1 variant was detected (Figure 3), and no binding of any Ig neurexin fusion protein to HEK293 cells that do not express a neuroligin 1 was observed (data not shown). These data agree well with the affinity chromatography and SPR results, except that the splice site B-positive variant of neuroligin 1 now also bound to neurexin 1β containing an insert in splice site 4 (and not only to neurexin 1β lacking an insert in splice site 4). This discrepancy is probably due to the fact that the alternative splicing of β-neurexins and the alternative splicing of neuroligins do not produce all-or-none effects but change the binding affinities to different extents, and that the affinity chromatography experiments are more stringent, while the SPR and cell surface binding experiments are more sensitive.

By what mechanism does the splice site B insert regulate binding of neuroligin 1 to neurexins? To address this question, we investigated whether neurexin 1α and 1β bind to neuroligin 1 via a similar interaction. In neurexin 1β, both the LG domain and a short β-specific N-terminal sequence are required for neuroligin 1 binding (Nguyen and Südhof, 1997). To dissect the binding site of neurexin 1α for neuroligin 1, we produced a series of Ig fusion proteins of α-neurexins that incorporate a subset of domains with selected splice variants (Figure 4A) and analyzed their binding to recombinant neuroligins using affinity chromatography (Figure 4B). Only neurexin 1α fragments that included both the third EGF-like sequence and the sixth LG domain were able to bind to neuroligin 1. Thus, in neurexin 1α and 1β, binding of neuroligin 1 is mediated by the combined action of an LG domain that is shared between neurexin 1α and 1β, and of a sequence N-terminal to the LG domain that differs between neurexin 1α and 1β.

We then examined how the insert sequence of splice site B restricts α-neurexin binding. Interestingly, this sequence includes an N-glycosylation site at asparagine

303 that is occupied by a complex oligosaccharide chain (Hoffman et al., 2004) and decreases the affinity of neuroligin 1 for insert-negative β-neurexins (Comoletti et al., 2003). To test whether N-glycosylation of the splice site B insert generally regulates neurexin binding, we investigated the effect of deglycosylation of neuroligin 1 on α-neurexin binding (Figure S4). We found that deglycosylation strongly activated neurexin 1α binding to neuroligin 1 containing an insert in splice site B, demonstrating that N-glycosylation at this site controls the binding specificity of neuroligin 1.

The definition of neuroligin 1 splice variants that bind either to only β-neurexins or to both α- and β-neurexins, provides an opportunity to test the functional significance of these interactions. We first tested both splice variants in the artificial synapse formation assay but detected no major difference between the two splice variants (Figure S5). We next transfected cultured hippocampal neurons with either EGFP (as a negative control) or EGFP-tagged neuroligin 1 containing (NL1-EGFP) or lacking (NL1ΔAB-EGFP) inserts in splice sites A and B. Four days later, the neurons were fixed, stained with antibodies to synapsin and MAP2 (as presynaptic and dendritic markers, respectively), and analyzed by confocal microscopy (Figure 5A). As expected, neuroligin 1-EGFPs were highly enriched in dendritic spines opposite to presynaptic terminals (arrows in Figure 5A), whereas EGFP not fused to neuroligin 1 was present in both dendrites and spines.

To quantitate the effects of the transfected proteins, we analyzed two parameters: the number of synapses and spines per dendrite length, and the size of presynaptic terminals and spines. These analyses were performed in multiple independent transfection experiments and "blindly" in that the experimenter was unaware of the nature of transfected proteins. Surprisingly, we found that, compared to EGFP transfection alone, neuroligin 1 containing splice site inserts induced a >2-fold increase in synapse numbers, whereas neuroligin 1 lacking splice site inserts was much less potent (Figure 5B). In contrast, neuroligin 1 lacking splice site inserts was significantly more effective in increasing the size of spines and presynaptic terminals than neuroligin 1 containing splice site inserts. The dramatic difference between the two splice variants of neuroligin 1 was not simply due to a difference in expression as evidenced by two facts: (1) the EGFP signal was similar, and (2) each of the two splice variants is more potent than the other in one of the two parameters analyzed. These data demonstrate that alternative splicing of neuroligin 1, presumably by preventing or promoting binding of α-neurexins in addition to β-neurexins, has a profound effect on *trans*-synaptic signaling.

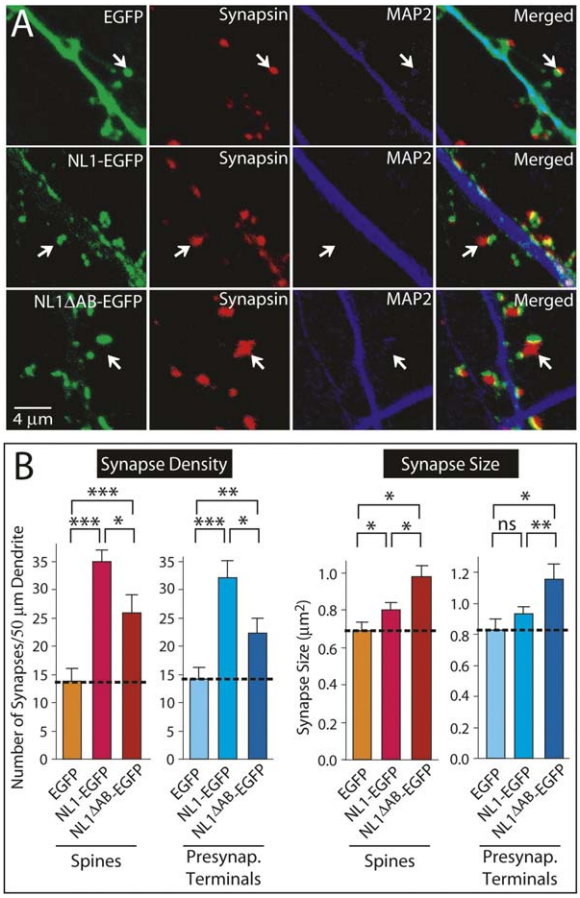

Figure 5. Effect of Neuroligin 1 Splice Variants that Bind Either Only to β-Neurexins or to Both α- and β-Neurexins in Transfected Neurons

(A) Representative fluorescence images of neurons transfected with EGFP-tagged neuroligin 1 containing (NL1-EGFP) or lacking (NL1ΔAB-EGFP) inserts in splice sites A and B, and of EGFP alone as a negative control. Images show the EGFP fluorescence (green), synapsin (red), and MAP2 immunofluorescence (blue), and the merged view of all three fluorescence pictures. Note that neuroligin 1 is exclusively present in spines, while EGFP is located in both dendrites and spines, and that the signals for neuroligins in spines and synapsin in nerve terminals are adjacent but not coincident (arrows). Calibration bar in bottom left corner applies to all panels.

(B) Quantitative analysis of the synapse density on dendrites of transfected neurons and of the synapse size on these dendrites. Both synapse density and size were measured by observers unaware of the transfected molecule using spine fluorescence (due to the neuroligin-EGFP fusion proteins) and presynaptic nerve terminal fluorescence (visualized by synapsin immunostaining). Asterisks above the bar diagrams indicate statistical significance of differences in pairwise comparisons between the three types of transfections (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001; ns, nonsignificant). Error bars represent means ± SEMs.

Discussion

In the present study, we show the following: (1) neurexin 1α tightly binds two cell surface proteins, dystroglycan, as shown previously (Sugita et al., 2001), and neuroligins 1–3 (Figure 1 and Figures S1 and S2); (2) the interaction of neurexin 1α and 1β with neuroligin 1 is regulated by alternative splicing at site B of neuroligin 1 in an all-or-none fashion, and by alternative splicing at site 4 of neurexin in a gradual fashion (Figure 2 and Figure S3); (3) alternative splicing at site B of neuroligin 1 controls α-neurexin binding by a glycosylation-dependent mechanism (Figure 2); and (4) neuroligin 1 splice variants that either bind only β-neurexins or both α- and β-neurexins have profoundly different effects on synapse size and density when expressed in neurons (Figure 5). This last point is particularly important because it indicates that presynaptic activation of α- and

β-neurexins by postsynaptic neuroligins has distinct functional consequences. Viewed together, our data reveal a general interaction of both α- and β-neurexins with neuroligins and suggest that this interaction represents a fundamental *trans*-synaptic adhesion mechanism. Our data indicate that the combination of neurexin and neuroligin variants at a synapse represents a *trans*-synaptic recognition code that shapes the properties of that synapse and the corresponding circuit.

The binding of neuroligins to α-neurexins was unexpected because earlier studies suggested that neuroligins only bind to β-neurexins (Ichtchenko et al., 1995, 1996; Nguyen and Südhof, 1997). This discrepancy is explained by the fact that we previously employed only the neuroligin 1 variant with an insert in splice site B that, as we confirmed here, is indeed specific for β-neurexins. In contrast, we now find that neuroligin 1 (and neuroligins 2 and 3) lacking an insert in splice site B avidly bind to both α- and β-neurexins (Figures 2 and 3; Figure S1). Consistent with the common binding of α- and β-neurexins to neuroligins, we found that neurexin 1α bound neuroligin 1 via the same LG domain that mediates neuroligin 1 binding in neurexin 1β (Figure 4).

It was previously shown in KO mice that α-neurexins are essential for synaptic transmission even when β-neurexins are present (Missler et al., 2003; Zhang et al., 2005). Thus, the functions of α- and β-neurexins do not completely overlap. This conclusion is reinforced by our present finding that neuroligin 1 variants that bind either only β-neurexins or both α- and β-neurexins exert differential effects on synapse formation. At the same time, both neuroligin 1 splice variants cause dramatic changes in synapse formation, indicating that both α- and β-neurexins act as *trans*-synaptic cell adhesion molecules by binding to neuroligins. The fact that α-neurexins perform additional functions beyond the common role of α- and β-neurexins suggests an explanation for the divergent functions observed in α-neurexin KO mice versus β-neurexin overexpression studies, namely that α- and β-neurexins have a general role in synapse formation mediated by neuroligin binding, a role that is still operative in the α-neurexin KO mice because these mice continue to express β-neurexins, and that α-neurexins have a special additional function in synapse organization. The special function of α-neurexins is presumably executed via other ligands, possibly dystroglycan and/or neurexophilins, or as yet unidentified cell surface proteins. This function could consist of a role in synapse formation as suggested by RNA interference experiments of neuroligins (Chih et al., 2005), although the lack of redundancy among neuroligins in these experiments is puzzling considering the redundancy among α-neurexins (Missler et al., 2003). Alternatively, this shared function could specify the balance between excitatory and inhibitory synapses as suggested by transfection experiments (Prange et al., 2004).

It is remarkable that the insertion or exclusion of only eight residues in splice site B of neuroligin 1 activates or blocks α-neurexin binding but leaves β-neurexin binding intact. We showed that in α- and β-neurexins the neuroligin binding sites include the same C-terminal LG domain but require different N-terminal sequences, suggesting that these N-terminal sequences are responsible for the distinct neuroligin 1 binding properties of α- and β-neurexins. In neuroligin 1, the splice site B insert appears to block α-neurexin binding via glycosylation because the insert sequence contains a conserved N-glycosylation site, and because deglycosylation of neuroligin 1 activates α-neurexin binding (Figure S4). On top of the regulation of α- and β-neurexin binding by alternative splicing of neuroligin 1, the affinity of α- and β-neurexin binding to neuroligins is modulated ~2-fold by alternative splicing of neurexins at splice site 4 (Figure 2). It thus seems likely that *trans*-synaptic signaling by the neurexin/neuroligin complex may be controlled by a hierarchy of regulatory events: transcription (α- versus β-neurexins, different types of neuroligins), alternative splicing of neuroligin 1 at site B, and alternative splicing of neurexins at site 4. Such a hierarchy of regulatory events would be well suited to contribute to shaping the properties of synaptic circuits, a possibility that will have to be explored in future experiments using genetic approaches that allow studying more physiological changes.

Experimental Procedures

Production of Recombinant Neurexins and Neuroligins

Neurexins

Neurexins were produced as Ig fusion proteins encoded by a series of constructs (Figure 4A) that were largely reported previously (Ushkaryov et al., 1994; Sugita et al., 2001; Missler et al., 1998a) and are described in detail in the Supplemental Data. Ig proteins were purified on protein-A Sepharose from the media of transfected COS cells and either used directly or eluted with 0.1 M glycine (pH 2.2).

Neuroligins

Neuroligins were produced as a series of secreted truncated proteins (Figure 2A) with an N-terminal Flag tag essentially as described (Comoletti et al., 2003), or as full-length proteins lacking or containing EGFP inserted into the cytoplasmic sequence. See the Supplemental Data for a detailed account of the constructs and methods used.

Brain Affinity Chromatography

Brain affinity chromatography was performed with brain proteins solubilized in 1% Triton X-100 essentially as described (Ichtchenko et al., 1995) with modifications (see Supplemental Data). Proteins bound to the neurexin or neuroligin affinity matrices were identified by mass spectrometry analysis (UTSouthwestern Protein Identification Facility), and identifications were confirmed by immunoblotting.

Pull-Down Assays

Pull-down assays were performed with immobilized Ig neurexins or Flag-tagged neuroligins essentially as described (Sugita et al., 2001).

SPR Analysis of the NL1-NX1α Complex

NL1 binding was analyzed at 25ºC using 10 mM HEPES buffer (pH 7.4) containing 2 mM $CaCl_2$ and 0.005% (v/v) surfactant P20, on a BIAcore 3000 apparatus. Protein A (Sigma) was covalently bound to the carboxymethylated dextran matrix of a CM5 chip (BIAcore, Uppsala, Sweden), and IgG-neurexin was indirectly captured by protein A via its IgG portion ~10,000 resonance units (RU). A separate reference surface containing protein A was coupled with IgC protein. Reference data were subtracted from the sample flow channel to obtain specific NX1α binding. NL1 was injected at concentrations ranging from 3000 to 12 nM in 3-fold dilution.

Neurexin Cell Surface Binding Assays

Transfected HEK293T cells expressing full-length neuroligins in all four splice combinations were incubated in DMEM containing 20 mM HEPES-NaOH (pH 7.4), 0.1% BSA, and 0.15 μM of either

IgC, IgN1α-1, IgN1α-41, IgN1β-1, or IgN1β-3 for 16 hr at 4ºC, washed, and fixed with 4% paraformaldehyde in the absence of detergent. Cells were then reacted with mouse M2 Flag antibody, and bound Ig fusion proteins and Flag antibodies were visualized with secondary antibodies and analyzed by confocal microscopy.

Primary Neuronal Culture, Transfection, and Immunocytochemistry

Primary hippocampal neuronal cultures from newborn (P1) rats (Kavalali et al., 1999) were transfected with NL1-EGFP, NL1-ΔAB-EGFP, and EGFP constructs at 10 days in vitro (DIV) and analyzed at 14 days DIV by immunofluorescence staining for synapsin and MAP2 (see Supplemental Data). Stacks of z-section images obtained by confocal microscopy were converted to maximal projection images and analyzed blindly using NIH Image/ImageJ program, with area size, fluorescent intensity, and density of spines and presynaptic terminals per 50 μm of dendrite measured using the "Analyze particle" module. Statistical significance was determined by Student's t test.

Miscellaneous

SDS-PAGE and immunoblotting were performed using standard procedures; most antibodies employed were previously characterized (Sugita et al., 2001; Ichtchenko et al., 1995; Song et al., 1999; Biederer et al., 2002; Ushkaryov et al., 1992). To achieve deglycosylation, 10–15 μg of NL1 in 200 μl was incubated with PNGase F (Glyko, Novato, CA) for 10 hr at 37ºC.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and five supplemental figures and can be found with this article online at http://www.neuron.org/cgi/content/full/48/2/229/DC1/.

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