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Alternative Splicing Controls Selective *Trans*-Synaptic Interactions of the Neuroligin-Neurexin Complex

Report

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Summary

Formation of synapses requires specific cellular interactions that organize pre- and postsynaptic compartments. The neuroligin-neurexin complex mediates heterophilic adhesion and can trigger assembly of glutamatergic and GABAergic synapses in cultured hippocampal neurons. Both neuroligins and neurexins are encoded by multiple genes. Alternative splicing generates large numbers of isoforms, which may engage in selective axo-dendritic interactions. We explored whether alternative splicing of the postsynaptic neuroligins modifies their activity toward glutamatergic and GABAergic axons. We find that small extracellular splice insertions restrict the function of neuroligin-1 and -2 to glutamatergic and GABAergic contacts and alter interaction with presynaptic neurexins. The neuroligin isoforms associated with GABAergic contacts bind to neurexin-1α and a subset of neurexin-1βs. In turn, these neurexin isoforms induce GABAergic but not glutamatergic postsynaptic differentiation. Our findings suggest that alternative splicing plays a central role in regulating selective extracellular interactions through the neuroligin-neurexin complex at glutamatergic and GABAergic synapses.

Introduction

The nervous system consists of an intricate network of neurons that are connected by synaptic junctions. Understanding how selective wiring between specific synaptic partners is achieved represents a major question in neurodevelopment. Sperry proposed that chemoaffinity labels presented at the surface of individual cells might direct selective neuronal growth and connectivity (Sperry, 1963). Adhesion molecules represent attractive candidates for mediating such selective cell-cell interactions (Benson et al., 2001; Scheiffele, 2003; Yamagata et al., 2003; Waites et al., 2005). In particular, there has been considerable interest in gene families comprising large numbers of adhesive protein isoforms that could encode selective axon-target interactions at different synapses (Missler and Südhof, 1998; Schmucker and Flanagan, 2004; Weiner et al., 2005).

The neuroligin-neurexin protein complex is a heterophilic adhesion system that might mediate selective *trans*-synaptic interactions. Neurexins represent one of the most diverse protein families in the mammalian nervous system, with over 1000 isoforms expressed from three neurexin genes (*NRXN1*, *-2*, and *-3*) (Ushkaryov et al., 1992; Ullrich et al., 1995). This molecular diversity is generated by expression from two alternative promoters that yield a long α- and a shorter β-neurexin transcript and by alternative splicing at up to five sites in the neurexin pre-mRNA (splice sites 1 through 5) (Missler et al., 1998). Biochemical fractionation and immunolocalization studies with pan-neurexin antibodies revealed a concentration of neurexins at synapses in the hippocampus, suggesting that they mediate synaptic interactions (Ushkaryov et al., 1992; Butz et al., 1998; Dean et al., 2003). Neuroligins were identified as splice isoform-specific neurexin ligands that are encoded by five genes (*NLGN1*, *-2*, *-3*, *-4*, and *-4Y*) (Ichtchenko et al., 1995, 1996; Bolliger et al., 2001). So far, only NL1 and -2 have been analyzed in detail at the cellular level. Immunolocalization studies suggested that NL1 is primarily localized to the postsynaptic side of glutamatergic synapses, whereas NL2 is preferentially targeted to GABAergic synapses (Song et al., 1999; Varoqueaux et al., 2004). Additional studies revealed that a fraction of endogenous NL1 might be localized to GABAergic synapses (Levinson et al., 2005). Overexpression and loss-of-function experiments in cultured neurons revealed that NL1 and NL2 isoforms can promote the assembly of glutamatergic and GABAergic synapses by bidirectional signaling in vitro (Scheiffele et al., 2000; Graf et al., 2004; Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005; Sara et al., 2005). These experiments highlighted a potent activity of the neuroligin-neurexin complex in organizing pre- and postsynaptic domains through *trans*-synaptic interactions.

Neuroligins are alternatively spliced in their ectodomains. Inclusion of short alternative exons (20 and 9 amino acids in length) at two sites (designated A and B, respectively) generates four potential NL1 variants [NL1(−), NL1A, NL1B, and NL1AB; see Figure 1] (Ichtchenko et al., 1996). NL2 undergoes a similar splicing event at the first site, thereby generating two different isoforms [NL2(−) and NL2A]. When tested in synapse-induction assays with heterologous cells, all NL1 isoforms have the ability to induce clustering of synaptic vesicles in pontine axons (Scheiffele et al., 2000). A more recent study reported that inclusion of a splice insertion at the B site in NL1 alters the interaction with neurexins (Boucard et al., 2005). Specifically, the B insertion in NL1 prevents binding to those β-NRX isoforms that contain a splice insertion at site 4. Moreover, the B insertion prevents binding to α-NRX isoforms (Boucard et al., 2005). However, the cellular consequences of these selective interactions are incompletely understood.

One important question that emerged from the previous work is how the specificity of neuroligin isoforms for glutamatergic and GABAergic synapses is encoded. In principle, such specificity could arise from selective extracellular interactions between specific pairs of neuroligin and neurexin isoforms. In an alternative model, extracellular neuroligin-neurexin interactions might be rather unselective, and neuroligin isoforms could be

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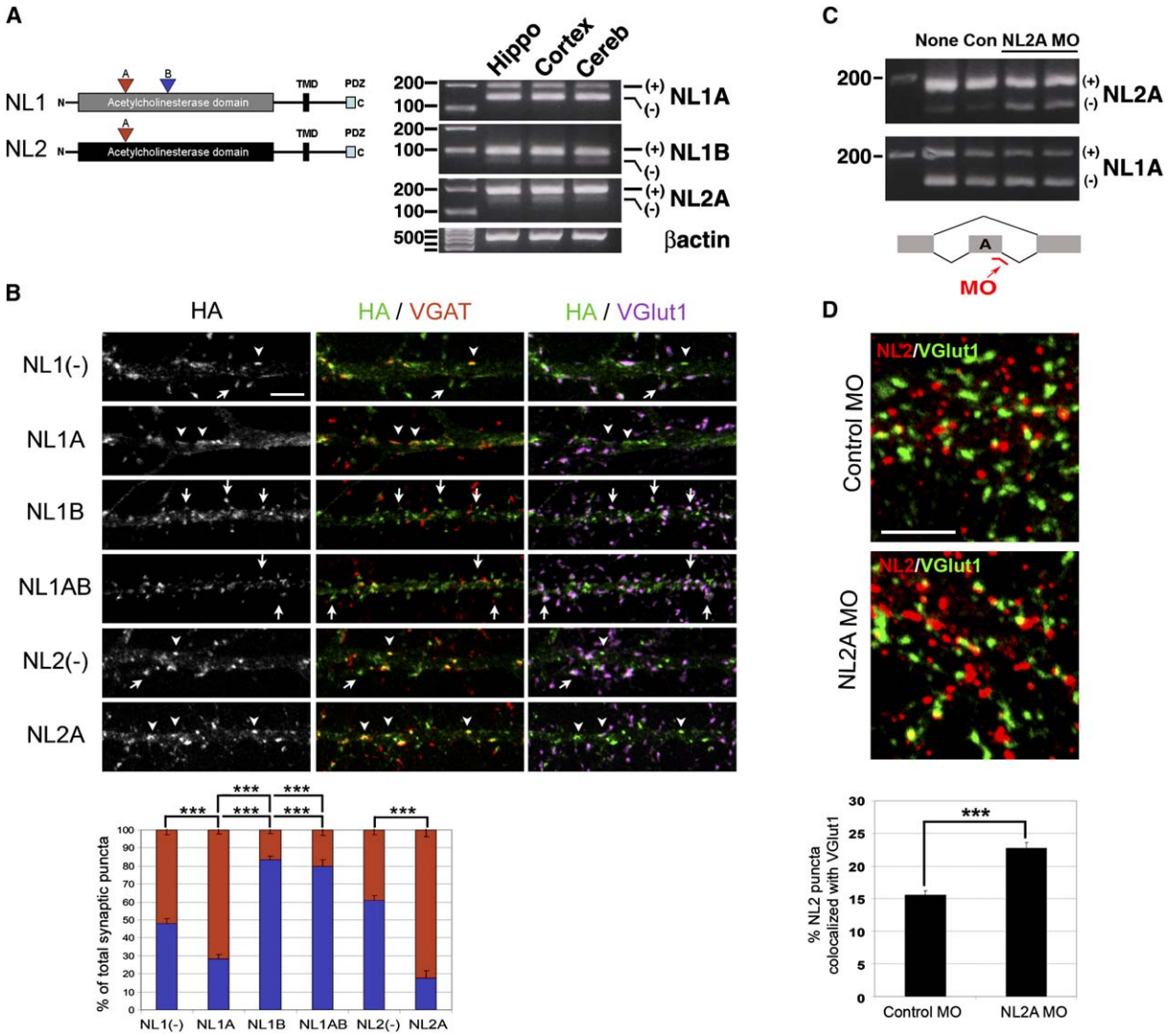

Figure 1. Alternative Splicing of Neuroligins Alters Targeting to Glutamatergic and GABAergic Synaptic Contacts

(A) Diagram of insertions A and B in NL1 and -2. RT-PCR analysis with primers flanking the splice insertion sites on RNA isolated from rat hippocampus, cortex, and cerebellum. β-actin was amplified as a positive control. Products containing (+) and lacking (−) the splice insertion are marked. Molecular weight markers are in base pairs. (B) Dendritic segments of cultured hippocampal neurons expressing low levels of transfected neuroligin splice variants. At 10 DIV, cells were transfected with NL1 lacking splice insertions A and B [NL1(−)], containing a single insertion (NL1A and NL1B), containing both splice insertions (NL1AB), or with constructs for NL2 lacking or containing a splice insertion [NL2(−) and NL2A, respectively]. Three days after transfection, cells were triple immunostained with antibodies to the HA-eptiope on the neuroligin isoforms (green), VGAT (red), and VGlut1 (magenta). Some immunoreactive dendritic spine heads are marked with arrows, and shaft synapses are marked with arrowheads. The quantitation shows the percent association of puncta formed by the neuroligin splice variants with VGAT- and VGlut1-positive structures (shown in red and blue, respectively; n = 10 cells and >2000 puncta for each condition, ANOVA $p < 0.0001$). Scale bar, 5 μm. (C) Inclusion of the NL2 splice insertion A was suppressed by a morpholino oligonucleotide (MO) complementary to the splice donor site of the exon encoding the NL2A insertion. RT-PCR products from two independent culture wells (NL2A MO) are compared to an untreated (None) and a culture control MO (Con). No effect on splicing of NL1A was observed (lower panel). (D) Hippocampal neurons were transfected with control morpholino oligos (MO) or NL2A-specific MO and stained with NL2 (red) and VGlut1 (green) antibodies (n = 10 fields for each condition, t test $p < 0.0001$). Scale bar, 5 μm. Error bars show mean ± SD.

recruited to glutamatergic versus GABAergic sites by interaction with synapse-specific cytoplasmic scaffolds. So far, there is only direct support for the latter, cytoplasmic specificity mechanism: overexpression of PSD-95, a glutamatergic scaffolding protein, recruits endogenous NL2 protein to glutamatergic synapses, and similarly, expression of PSD-95 has been shown to restrict NL1 to glutamatergic sites (Graf et al., 2004; Prange et al., 2004; Levinson et al., 2005). However, the role of individual splice isoforms of neuroligins in synaptic selectivity has not been analyzed.

In the present study, we explored whether specific neuroligin splice isoforms differ in their targeting to glutamatergic or GABAergic axo-dendritic contacts and whether specific neuroligin and neurexin splice isoforms exhibit functional selectivity. We find that alternative splicing in the NL1 extracellular domain controls the localization and synapse-inducing activity of the protein

toward glutamatergic and GABAergic axons. We also find that this differential activity of neuroligin proteins is mediated through differential interaction with specific neurexin splice variants. These data provide strong evidence that the molecular diversity of the neuroligin and neurexin gene families mediates selective interactions at CNS synapses.

Results

Expression of Neuroligin Splice Isoforms

To begin analyzing the selectivity of neuroligin-neurexin isoform interactions, we examined the expression of NL1 and -2 isoforms by RT-PCR. NL1 pre-mRNA can be alternatively spliced in sequences encoding the extracellular domain at two positions, designated A and B (Figure 1A) (Ichtchenko et al., 1996; Scheiffele et al., 2000). Analysis with primers flanking the splice insertion sites indicated the presence of mRNAs containing and lacking splice insertion A and B in different brain areas (Figure 1A). While mRNAs with and without the A insertion were detected at similar levels, there was only a small but significant pool of NL1 mRNA lacking the B insertion. For NL2, we observed RT-PCR products containing and lacking the splice insertion in site A, with the A(+) form being more abundant than the A(−) form (Figure 1A). NL2-specific primers flanking the position analogous to splice site B in NL1 did not amplify an insertion-containing product (data not shown), consistent with the previous suggestion that NL2 is not alternatively spliced at this position (Ichtchenko et al., 1996). RT-PCR performed with RNA isolated from cultured hippocampal neurons revealed similar incorporation of A and B in both neuroligins (data not shown, but see Figure 1C below). This suggests that multiple NL1 and -2 splice variants are expressed in vitro and in vivo.

It is unknown whether alternative splicing in the extracellular domain alters neuroligin localization. To address this question, we established conditions to express epitope-tagged forms of all NL1 and -2 splice variants at low levels in cultured hippocampal neurons. Under these conditions, neuroligin expression did not result in a significant increase in the density of presynaptic terminals formed on the transfected cells ($n = 10$, ANOVA $p > 0.05$), in contrast to significant overexpression, which results in a marked increase of presynaptic terminal density (Dean et al., 2003; Prange et al., 2004; Boucard et al., 2005; Chih et al., 2005). The synaptic localization of the dendritic neuroligin isoforms was then assessed by triple staining with antibodies to the HA-epitope tag in neuroligin and antibodies to the presynaptic markers of glutamatergic (VGlut1) and GABAergic (VGAT) synapses. Figure 1B shows representative images of the localization of the epitope-tagged proteins along segments of dendrites. As judged by overlap with the combination of both presynaptic markers, about 70% of punctate structures formed by all postsynaptic neuroligin isoforms were concentrated at synaptic contacts. However, the NL1 splice isoforms greatly differed in their apposition to vGlut1- and VGAT-positive terminals (Figure 1B). NL1(−) that lacks both splice insertions showed comparable overlap with vGlut1- and VGAT-positive sites (VGlut1:VGAT, 48:52% ± 3%). NL1A that contains insertion A was preferentially targeted to VGAT-positive structures on the dendritic shaft (28:72% ± 2%). In contrast, NL1B was targeted to vGlut1-positive sites and was often concentrated in spine heads (83:17% ± 2%). Inclusion of both splice insertions (NL1AB) resulted in a protein that was targeted to vGlut1-positive sites like NL1B (80:20% ± 3%), suggesting that the presence of the B insertion overrides the activity of the A insertion (Figure 1B).

Synaptic localization of NL2 splice variants followed a similar pattern. NL2 lacking a splice insertion was similarly distributed over vGlut1- and VGAT-positive puncta, whereas inclusion of insertion A increased localization at VGAT-positive sites (Figure 1B). Interestingly, cells expressing NL2A showed a slight decrease in the density of VGlut1-positive puncta (vGlut1 puncta/10 μm dendrite: GFP = 5.62 ± 1.14, NL2A = 3.28 ± 1.16, $n = 10$, $p < 0.05$, no statistically significant change in VGAT puncta density). This suggests that subtly altering the abundance of this neuroligin splice variant may alter the ratio of glutamatergic and GABAergic contacts formed on the cell. However, low-level expression of any of the other neuroligin splice variants did not result in statistically significant changes in the glutamatergic:GABAergic contact ratio under our experimental conditions.

To test directly whether the A insertion in endogenous NL2 contributes to the selective association of the protein with GABAergic contacts, we used an antisense morpholino-oligonucleotide approach to induce exon skipping (Schmajuk et al., 1999; Gebski et al., 2003). We designed a morpholino oligo that targets the splice donor side of the exon encoding insert A (Figure 1C). Delivery of this oligo into cultured hippocampal neurons specifically reduced inclusion of the A sequences in the NL2 mRNA but did not affect the A insertion in NL1 (Figure 1C). Importantly, in neurons treated with the NL2A-specific morpholino oligonucleotide, the endogenous NL2 showed increased association with the glutamatergic marker vGlut1 (Figure 1D). This further supports an important function of the A insertion in the synaptic specificity of NL2. In summary, these findings suggest a critical role for alternative splicing in the association of the NL1 and -2 isoforms with glutamatergic and GABAergic synaptic terminals in hippocampal neurons.

Neuroligin Splice Variants Have Different Synapse-Inducing Activities

To test whether the different neuroligin splice isoforms have selective activities in inducing the assembly of glutamatergic or GABAergic presynaptic terminals, we overexpressed them in cultured hippocampal neurons. As in the localization experiments, NL1 splice variants differed in the induction of glutamatergic and GABAergic terminals (Figure 2). NL1(−) and NL1A significantly increased the density of both glutamatergic and GABAergic terminals. In contrast, NL1B and NL1AB greatly increased glutamatergic terminal density but had little effect on GABAergic terminals. This suggests that the B insertion sequence prevents activity of NL1 toward GABAergic axons. Previous work demonstrated that overexpression of NL1AB promotes the formation of dendritic spines (Boucard et al., 2005; Chih et al., 2005; Sara et al., 2005). However, the selectivity of NL1AB for induction of glutamatergic synapses is not a secondary consequence of alterations in dendritic morphology and cell-cell contact.

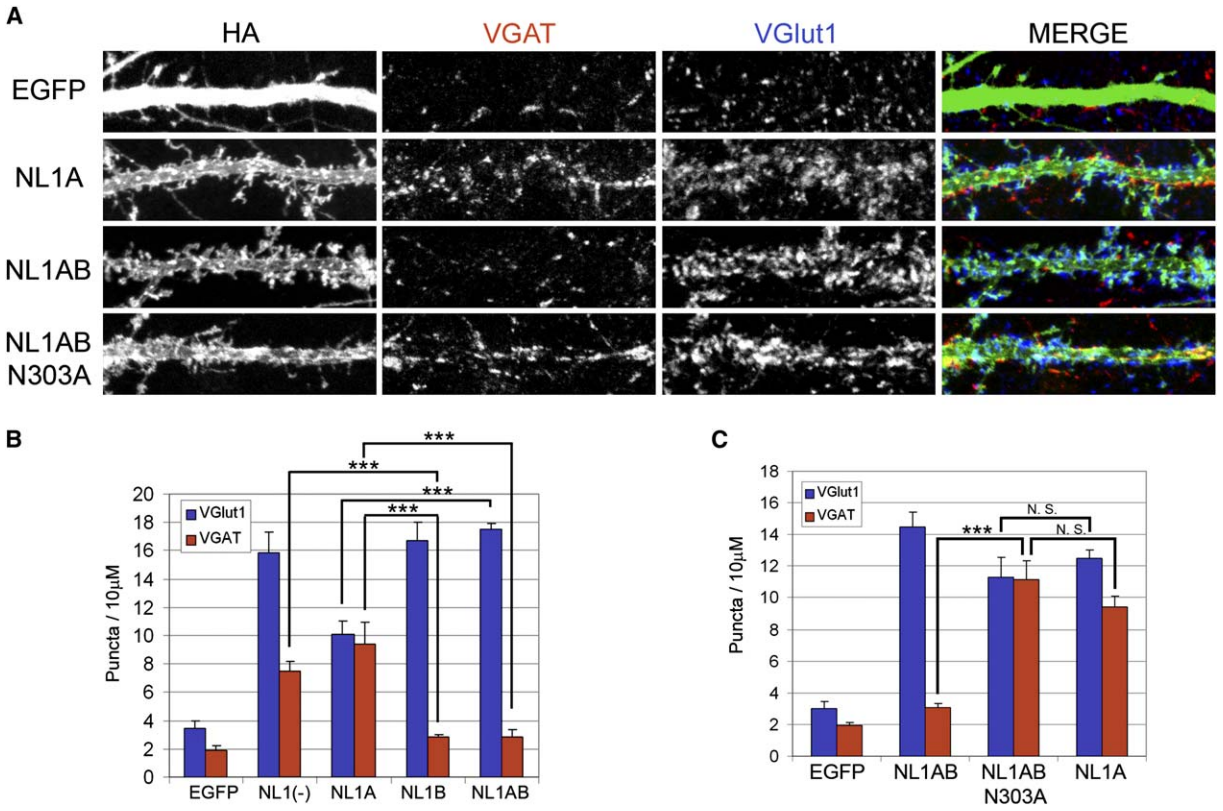

Figure 2. Presence of the B Insertion in Neuroligin-1 Controls Activity in Glutamatergic Synapse Induction

(A) Dendritic segments of cultured hippocampal neurons overexpressing NL1 variants. Cultures were transfected at 10 DIV and analyzed 3 days later. Cells were immunostained with antibodies to the HA-epitope on the neuroligin isoforms (green), VGAT (red), and VGlut1 (blue). Scale bar, 5 μm. (B) Density of presynaptic vGlut1- and VGAT-positive puncta on cells overexpressing EGFP, NL1(−), NL1A, NL1B, or NL1AB (n = 10 cells for each condition, ANOVA $p < 0.0001$). (C) Glycosylation of the B insertion is essential for selective activity at glutamatergic synapses. The synapse-inducing activity of the mutant NL1AB (N303A) in which the asparagine of the N-glycosylation site was mutated to alanine was compared to NL1AB and NL1A (n = 10 cells for each condition, ANOVA $p < 0.0001$). Error bars show mean ± SD.

A large proportion of the NL1AB-induced vGlut1-positive sites were localized to dendritic shafts, indicating that it is the identity of the extracellular domain and not the presence of spines that directs the selective assembly of glutamatergic terminals in response to NL1AB. Boucard et al. demonstrated that synapsin-positive vesicle clusters induced by overexpression of NL1(−) in cultured hippocampal neurons were enlarged as compared to NL1B (Boucard et al., 2005). Using neurotransmitter-specific vesicle markers vGlut1 and VGAT, we found a significant enlargement of both VGAT- and vGlut1-positive puncta in NL1(−)-expressing cells, whereas NL1B primarily increased vGlut1-positive puncta size but had little effect on VGAT-positive puncta (see Figure S1 in the Supplemental Data available online). This further confirms a synapse-specific activity of the NL1 variants.

The B insertion sequence in NL1 consist of only nine amino acids but contains a consensus sequence for N-glycosylation that is modified with complex-type glycans (Comoletti et al., 2003; Hoffman et al., 2004). To test whether the presence of N-glycans in the B insertion was critical for restricting NL1 function to glutamatergic synapses, we mutated asparagine 303 to alanine and expressed the mutant protein in dissociated hippocampal neurons (Figure 2). Removal of the N-glycosylation site greatly increased the activity of NL1AB for induction of VGAT-positive presynaptic terminals, thereby shifting the synapse-inducing activity of NL1AB to a pattern similar to NL1A (Figure 2B). Most likely, the large carbohydrate structure in the B splice insert inhibits NL1B activity toward GABAergic synapse induction, possibly by hindering interactions with ligands. Such a mechanism might also explain why the presence of the B insertion is dominant over the A insertion, as observed in the low-expression experiments (Figure 1).

The NL2(−) and NL2A isoforms showed comparable induction of vGlut1 and VGAT-positive terminals when overexpressed (Figure 3).To further test the relevance of the B insertion for the selectivity for glutamatergic synapses, we transplanted the B sequence of NL1 into NL2, which normally is not alternatively spliced at this position. When tested in the synapse-induction assay in hippocampal neurons, the resulting NL2B and NL2AB variants lost their ability to induce GABAergic presynaptic terminals (Figure 3). This strongly supports the idea that the presence of a splice insertion at the B site restricts the activity of neuroligins to glutamatergic synapses.

Specific Neurexin Variants with Selective Activity for GABAergic Synapse Formation

We hypothesized that the differential synapse-inducing activities of neuroligin splice variants might be caused

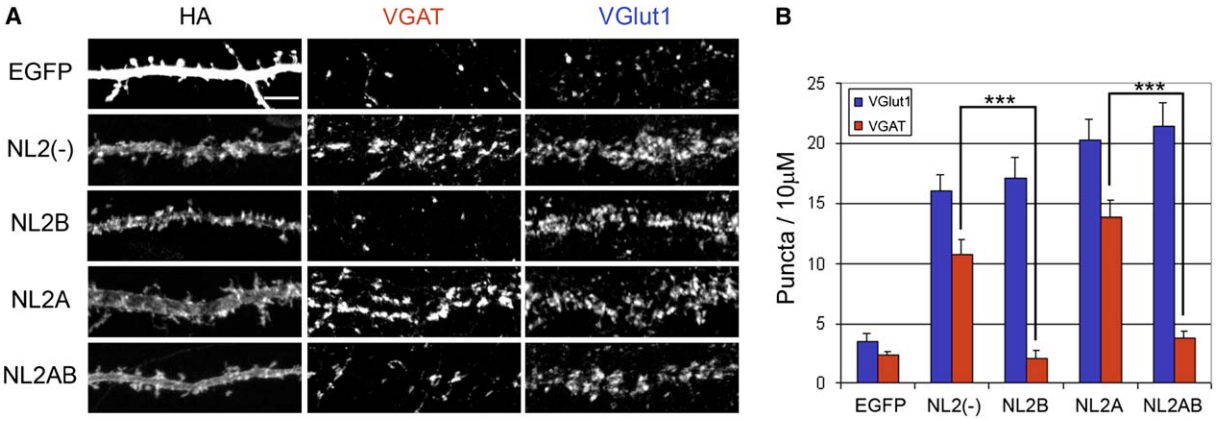

Figure 3. Introduction of the B Insertion into Neuroligin-2 Prevents Induction of GABAergic Presynaptic Terminals

(A) The NL2 splice variants NL2(−) and NL2A and the artificial variants into which the B exon from NL1 had been inserted (NL2B and NL2AB) were overexpressed in cultured hippocampal neurons (10 + 3 DIV). Cells were immunostained with antibodies to the HA-epitope on the neuroligin isoforms, VGAT, and VGlut1. Scale bar, 5 μm. (B) Density of presynaptic puncta on overexpressing cells was quantified for ten cells as described in Experimental Procedures (n = 10 cells for each condition, ANOVA $p < 0.0001$). Error bars show mean ± SD.

by differential interaction with neurexin isoforms expressed on glutamatergic and GABAergic axons. To probe whether endogenous neurexins are indeed recruited by neuroligin isoforms, we performed immunohistochemistry with pan-neurexin antibodies. We observed a strong increase in the neurexin concentration at glutamatergic and GABAergic terminals in response to expression of NL1AB and NL2A, respectively (Figure S2). To begin to explore differential binding of neurexins to neuroligin variants, we focused on splice site 4 of NRX1, which has been shown to be a critical regulator of neuroligin-neurexin interactions (Ichtchenko et al., 1995; Boucard et al., 2005; Graf et al., 2006). When tested in binding assays with neuroligin-expressing cells, recombinant NRX1β containing and lacking an insertion in site 4 [NRX1β4(+) and -4(−)] showed differential interaction with neuroligins. While NRX1β4(−) bound similarly to cells expressing any of the NL1 and -2 splice variants, the isoform containing an insertion in site 4 [NRX1β4(+)] did not bind to NL1B and NL1AB, but efficiently interacted with NL1(−), NL1A, NL2(−), and NL2A (Figure S3). These findings confirmed a recent report that demonstrated that the presence of splice insertion B in NL1 prevents interaction with NRX1β4(+) (Boucard et al., 2005). In the same study, Boucard et al. also showed that NL1 lacking insertion B can interact with NRX1α, regardless of whether the neurexin contains or lacks splice insertion 4. This selectivity imposed by the B insertion in NL1 requires the N-glycosylation site in B (Boucard et al., 2005). Based on these biochemical interactions and the selective activities of neuroligin isoforms observed in our cellular assays, we predicted that GABAergic axons should employ neurexin variants that bind to NL1A and NL2A but not to NL1AB and NL1B. Accordingly, NRX1β4(+) and α-neurexins are candidate presynaptic binding partners for neuroligins at GABAergic synapses.

To probe for a selectivity in synapse induction by α- versus β-neurexins and neurexins alternatively spliced at site 4, we examined the NRX1α4(−), NRX1β4(−), and NRX1β4(+) variants in more detail. We employed a mixed-culture assay that measures the assembly of postsynaptic structures in response to contact with non-neuronal cells transfected with a single neurexin isoform (Graf et al., 2004). The differential binding of NRX1β4(+) and NRX1α4(−) to the GABAergic neuroligins predicts that these neurexins should exclusively promote GABAergic postsynaptic differentiation. We expressed NRX1β4(+), NRX1α4(−), and as a control, NRX1β4(−) in COS cells and added these cells to cultured hippocampal neurons. As previously shown (Graf et al., 2004; Nam and Chen, 2005), NRX1β4(−) induced glutamatergic and GABAergic postsynaptic structures positive for PSD-95 and gephyrin, respectively (Figure 4). In contrast, NRX1β4(+) and NRX1α4(−) exclusively triggered the assembly of a gephyrin-containing postsynaptic scaffold. These structures were VGAT negative, confirming that they were induced by the heterologous cells and did not represent pre-existing neuron-neuron contacts. Moreover, the GABAergic postsynaptic sites recruited endogenous NL2 (Figure S4), which according to our RT-PCR analysis is the most abundant binding partner for these neurexin isoforms in dissociated hippocampal neurons. These findings suggest a selective activity of two classes of neurexins in the induction of GABAergic synapses in hippocampal neurons: α-neurexins and the subset of β-neurexins containing a splice insertion in site 4.

To confirm a differential activity of neurexin splice variants, we used the extracellular domain of NRX1β as a blocking reagent. In previous studies, we demonstrated that a recombinant NRX1β4(−)-Fc fusion protein blocked glutamatergic synapse formation between cerebellar neurons, presumably by competing with the endogenous neurexin isoforms for binding partners (Scheiffele et al., 2000). In the same assay, NRX1β4(+)-Fc did not show any effect on glutamatergic synapse assembly. When applied to hippocampal neurons, NRX1β4(−)-Fc reduced the number of vGlut1- and VGAT-positive puncta (Figure 4E). In contrast, NRX1β4(+)-Fc selectively reduced the density of VGAT-positive terminals but had no effect on the density of vGlut1-positive puncta. This further supports an isoform-specific role for neurexins in the assembly of GABAergic terminals.

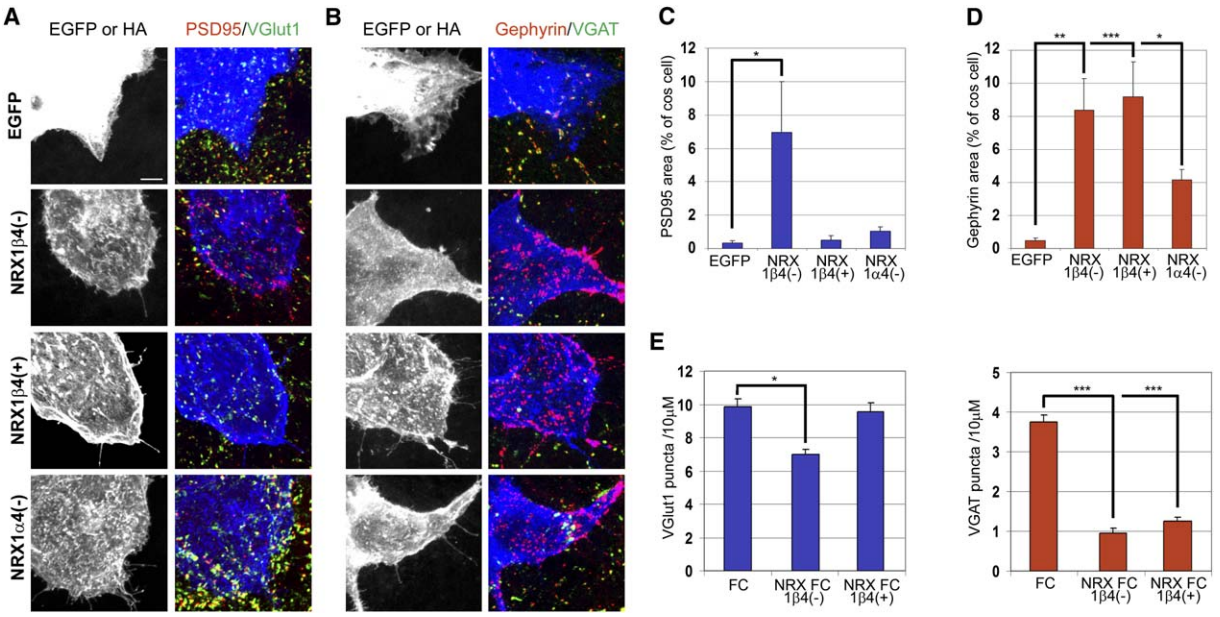

Figure 4. Selectivity of Neurexin-1 Isoforms in the Induction of Postsynaptic Differentiation

(A and B) COS cells expressing EGFP, NRX1β4(−), NRX1β4(+), or NRX1α4(−) were added to cultured hippocampal neurons at 10 DIV and analyzed 3 days later. The neurexin variants were detected with antibodies to the HA-epitope tag fused to the protein (blue) and protein accumulation in neurons was detected with antibodies to PSD-95 (in [A]) or gephyrin (in [B]) to mark glutamatergic and GABAergic postsynaptic structures, respectively (red). Postsynaptic structures in contact with the COS cells that do not contain presynaptic markers (VGlut1 or VGAT) are visualized as pink in the merge image. Scale bar, 10 μm. (C and D) Quantitation of clusters of postsynaptic markers PSD-95 and gephyrin induced by neurexin-expressing COS cells. The percentage of COS cell contact area was scored that is positive for the postsynaptic marker PSD-95 or gephyrin but negative for the corresponding presynaptic marker (VGlut1 or VGAT, respectively; n = 10 cells, for NRX1β4(+) PSD-95 $p = 0.28$; gephyrin $p < 0.001$). (E) Hippocampal neurons were transfected with an EGFP expression plasmid, and recombinant NRX1β4(−) and NRX1β4(+)-Fc fusion proteins were added to the culture medium at 6 DIV. The density of presynaptic vesicle clusters positive for VGlut1 and VGAT was quantified 6 days later (n = 10, ANOVA $p < 0.001$). Error bars show mean ± SD.

Discussion

Our studies reveal a central role for alternative splicing in the functional regulation of the neuroligin-neurexin complex at glutamatergic and GABAergic synapses. Selective interactions of each partner in this *trans*-synaptic complex are regulated by alternative splicing decisions. For GABAergic synapses, we observed a selective synapse-inducing activity for presynaptic NRX1α4(−) and NRX1β4(+) that can interact with postsynaptic NL1(−), NL1A, NL2(−), and NL2A isoforms (with NL2A being the most abundant ligand in hippocampal neurons). In contrast, we discovered that the B insertion in NL1 variants NL1B and NL1AB restricts NL1 function to glutamatergic synapses where they can interact with β-neurexin variants that lack an insertion at site 4. These findings identify neuroligin and neurexin splice variants with selective functions at glutamatergic and GABAergic synapses. In addition to these synapse-selective isoforms, our analysis also revealed that there are neurexin and neuroligin isoforms such as NRX1β4(−), NL1(−), and NL2(−) that promote both glutamatergic and GABAergic synaptic differentiation in our assay systems.

The finding that a subset of neurexin isoforms shows selective activity in the induction of GABAergic synapses is of particular interest considering that only few transmembrane components of GABAergic synapses are known thus far (Moss and Smart, 2001). Our analysis with pan-neurexin antibodies directly demonstrates concentration of endogenous neurexins at GABA synapses. The functional assays identified two classes of neurexin variants that exclusively induce assembly of GABAergic postsynaptic sites: α-neurexins and the subset of β-neurexins containing the splice insertion at site 4. Consistent with an important function of α-neurexins in GABAergic synapse assembly, α-neurexin triple knockout mice show a 50% reduction in the density of GABAergic synapses in the neocortex (Missler et al., 2003). For NRX1β4(+), our data demonstrate that alternative splicing plays a central role in controlling the interaction with neuroligins and the functional selectivity. The 30 amino acid splice insertion in NRX1β4(+) converts the unselective NRX1β4(−) isoform into a protein with synapse-selective function. A recent study by Graf et al. suggested that the 4(+) insertion decreases but does not abolish the activity of NRX1β4(+) toward glutamatergic postsynaptic assembly as well as its binding to NL1AB (Graf et al., 2006). In contrast, our results as well as those of Boucard et al. suggest that the 4(+) isoform lacks significant affinity for NL1AB, and our functional assays do not show accumulation of postsynaptic glutamatergic components above background levels. These differences might be attributed to different protein expression levels in the gain-of-function assays. Most importantly, our loss-of-function experiments show that recombinant NRX1β4(+) perturbs GABAergic presynaptic vesicle clustering but does not alter accumulation of glutamatergic markers, therefore confirming a synapse-specific activity of this neurexin variant. With respect to the postsynaptic neuroligin isoforms, we

observed that presence of the 20 amino acid A insertion in the extracellular domain of NL1 and NL2 increases the association of the protein with GABAergic presynaptic terminals. How the presence of the A insertion alters interaction with specific neurexins at GABAergic synapses remains to be investigated.

While these experiments highlight the role for alternative splicing in controlling synapse-specific activities of neuroligin and neurexin isoforms, the *trans*-synaptic neuroligin-neurexin adhesion code is likely to be complex. The neurexin isoforms NRX1β4(+) and NRX1α4(−) are not restricted to inhibitory interneurons but are also expressed in glutamatergic cells (Ullrich et al., 1995). Moreover, α-neurexin triple knockout mice also show defects in glutamatergic transmission (Missler et al., 2003). This argues against a simple model where specific cell types would express only a single neurexin variant that is directly linked to the neurotransmitter phenotype. More likely, different neuronal populations might employ multiple neurexin isoforms that act in combination with other synapse-specific factors. Future work will be required to clarify this; however, our data strongly suggest that splice isoform-specific neuroligin-neurexin interactions are differentially employed at GABAergic and glutamatergic synapses.

For NL1 and NL2, previous in situ hybridization studies suggest that both isoforms are coexpressed in many neuronal cell types (Scheiffele et al., 2000; Varoqueaux et al., 2004), which is not surprising considering that most cells receive both glutamatergic and GABAergic inputs. However, additional analysis of NL1 and -2 splice isoform expression at the single-cell level might reveal whether specific variants are differentially employed at some synapses. The selective extracellular interactions mediated by specific neuroligin and neurexin splice isoforms are likely to be further regulated by the previously proposed cytoplasmic mechanisms in controlling neuroligin localization and function (Prange et al., 2004; Levinson and El-Husseini, 2005). We identified NL1(−) and NL2(−) as two neuroligin splice variants with little apparent preference for specific synaptic contacts. The function of these variants might be most strongly dependent on interactions with the cytoplasmic scaffold, e.g., PSD-95. In contrast, NL1B and NL1AB show little activity toward GABAergic presynaptic terminals in the synapse-induction assays; these isoforms therefore are unlikely to be regulated through a mechanism depending on interactions with the cytoplasmic postsynaptic scaffold.

Utilizing specific variants of a single synaptic adhesion system at glutamatergic and GABAergic synapses has some interesting implications. The divergent extracellular interactions may contribute to cell-cell recognition events, whereas common intracellular domains may couple these interactions to similar synapse assembly or maturation pathways. Our experiments show that alternative splicing of neuroligins regulates the specificity of their synapse-inducing activities toward glutamatergic and GABAergic axons. This mechanism may be employed to rapidly alter synaptic interactions in response to signaling, for example by switching NL1 association from glutamatergic to GABAergic synapses by exclusion of the B insertion. Further analysis of the significant number of additional neuroligin and neurexin isoforms should determine whether other selective interactions are encoded by this neuronal receptor system and whether such interactions also contribute to selective *trans*-synaptic interactions at different types of central synapses.

Experimental Procedures

Neuronal Culture Assays

Dissociated cultures of hippocampal neurons were prepared from E18 rats, plated on poly-D-lysine-coated glass coverslips at a density of 30,000/cm^2, and maintained in neurobasal medium with B27 supplement (Invitrogen). Transfections were carried out with Lipofectamine 2000 (Invitrogen) as described previously (Chih et al., 2005). For low expression of neuroligin splice forms, 75 ng of DNA was used per coverslip in a 24-well plate, for overexpression experiments, 150 ng of DNA (see Supplemental Data for details on the DNA vectors).

Morpholino oligonucleotides (MOs) used to increase skipping of the A insertion in NL2 were designed based on methods previously used for other target genes (Schmajuk et al., 1999; Gebski et al., 2003). The following MOs were used (GeneTools): NL2A, 5′-ATTTTT AAATCTACCTGTGTCTGGC-3′; negative control oligo, 5′-CCTCTT ACCTCAGTTACAATTTATA-3′. MOs were added to individual wells in a 24-well plate containing 500 μl of culture medium at final concentration of 10 μM with gentle mixing. Then 4 μl of Endoporter (GeneTools) was added to each well. Neurons were treated at 6 DIV and incubated for 4 days.

See Supplemental Data for additional information on histochemistry and image acquisition.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/51/2/171/DC1/.

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References

Benson, D.L., Colman, D.R., and Huntley, G.W. (2001). Molecules, maps and synapse specificity. Nat. Rev. Neurosci. *2*, 899–909.

Bolliger, M.F., Frei, K., Winterhalter, K.H., and Gloor, S.M. (2001). Identification of a novel neuroligin in humans which binds to PSD-95 and has widespread expression. Biochem. J. *356*, 581–588.

Boucard, A.A., Chubykin, A.A., Comoletti, D., Taylor, P., and Südhof, T.C. (2005). A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to alpha- and beta-neurexins. Neuron *48*, 229–236.

Butz, S., Okamoto, M., and Südhof, T.C. (1998). A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. Cell *94*, 773–782.

Chih, B., Engelman, H., and Scheiffele, P. (2005). Control of excitatory and inhibitory synapse formation by neuroligins. Science *307*, 1324–1328.

Comoletti, D., Flynn, R., Jennings, L.L., Chubykin, A., Matsumura, T., Hasegawa, H., Südhof, T.C., and Taylor, P. (2003). Characterization of the interaction of a recombinant soluble neuroligin-1 with neurexin-1beta. J. Biol. Chem. *278*, 50497–50505.

Dean, C., Scholl, F.G., Choih, J., DeMaria, S., Berger, J., Isacoff, E., and Scheiffele, P. (2003). Neurexin mediates the assembly of presynaptic terminals. Nat. Neurosci. *6*, 708–716.

Gebski, B.L., Mann, C.J., Fletcher, S., and Wilton, S.D. (2003). Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. Hum. Mol. Genet. *12*, 1801–1811.

Graf, E.R., Zhang, X., Jin, S.X., Linhoff, M.W., and Craig, A.M. (2004). Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell *119*, 1013–1026.

Graf, E.R., Kang, Y., Hauner, A.M., and Craig, A.M. (2006). Structure function and splice site analysis of the synaptogenic activity of the neurexin-1 beta lns domain. J. Neurosci. *26*, 4256–4265.

Hoffman, R.C., Jennings, L.L., Tsigelny, I., Comoletti, D., Flynn, R.E., Südhof, T.C., and Taylor, P. (2004). Structural characterization of recombinant soluble rat neuroligin 1: Mapping of secondary structure and glycosylation by mass spectrometry. Biochemistry *43*, 1496–1506.

Ichtchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Südhof, T.C. (1995). Neuroligin 1: A splice site-specific ligand for beta-neurexins. Cell *81*, 435–443.

Ichtchenko, K., Nguyen, T., and Südhof, T.C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. J. Biol. Chem. *271*, 2676–2682.

Levinson, J.N., and El-Husseini, A. (2005). Building excitatory and inhibitory synapses: Balancing neuroligin partnerships. Neuron *48*, 171–174.

Levinson, J.N., Chery, N., Huang, K., Wong, T.P., Gerrow, K., Kang, R., Prange, O., Wang, Y.T., and El-Husseini, A. (2005). Neuroligins mediate excitatory and inhibitory synapse formation: Involvement of PSD-95 and neurexin-1beta in neuroligin induced synaptic specificity. J. Biol. Chem. *280*, 17312–17319.

Missler, M., and Südhof, T.C. (1998). Neurexins: Three genes and 1001 products. Trends Genet. *14*, 20–26.

Missler, M., Fernandez-Chacon, R., and Südhof, T.C. (1998). The making of neurexins. J. Neurochem. *71*, 1339–1347.

Missler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R.E., Gottmann, K., and Südhof, T.C. (2003). Alpha-neurexins couple Ca2+ channels to synaptic vesicle exocytosis. Nature *423*, 939–948.

Moss, S.J., and Smart, T.G. (2001). Constructing inhibitory synapses. Nat. Rev. Neurosci. *2*, 240–250.

Nam, C.I., and Chen, L. (2005). Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. Proc. Natl. Acad. Sci. USA *102*, 6137–6142.

Prange, O., Wong, T.P., Gerrow, K., Wang, Y.T., and El-Husseini, A. (2004). A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc. Natl. Acad. Sci. USA *101*, 13915–13920.

Sara, Y., Biederer, T., Atasoy, D., Chubykin, A., Mozhayeva, M.G., Südhof, T.C., and Kavalali, E.T. (2005). Selective capability of SynCAM and neuroligin for functional synapse assembly. J. Neurosci. *25*, 260–270.

Scheiffele, P. (2003). Cell-cell signaling during synapse formation in the CNS. Annu. Rev. Neurosci. *26*, 485–508.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell *101*, 657–669.

Schmajuk, G., Sierakowska, H., and Kole, R. (1999). Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. J. Biol. Chem. *274*, 21783–21789.

Schmucker, D., and Flanagan, J.G. (2004). Generation of recognition diversity in the nervous system. Neuron *44*, 219–222.

Song, J.Y., Ichtchenko, K., Südhof, T.C., and Brose, N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. Proc. Natl. Acad. Sci. USA *96*, 1100–1105.

Sperry, R.W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA *50*, 703–710.

Ullrich, B., Ushkaryov, Y.A., and Südhof, T.C. (1995). Cartography of neurexins: More than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. Neuron *14*, 497–507.

Ushkaryov, Y.A., Petrenko, A.G., Geppert, M., and Südhof, T.C. (1992). Neurexins: Synaptic cell surface proteins related to the alpha- latrotoxin receptor and laminin. Science *257*, 50–56.

Varoqueaux, F., Jamain, S., and Brose, N. (2004). Neuroligin 2 is exclusively localized to inhibitory synapses. Eur. J. Cell Biol. *83*, 449–456.

Waites, C.L., Craig, A.M., and Garner, C.C. (2005). Mechanisms of vertebrate synaptogenesis. Annu. Rev. Neurosci. *28*, 251–274.

Weiner, J.A., Wang, X., Tapia, J.C., and Sanes, J.R. (2005). Gamma protocadherins are required for synaptic development in the spinal cord. Proc. Natl. Acad. Sci. USA *102*, 8–14.

Yamagata, M., Sanes, J.R., and Weiner, J.A. (2003). Synaptic adhesion molecules. Curr. Opin. Cell Biol. *15*, 621–632.