Binding Properties of Neuroligin 1 and Neurexin 1β Reveal Function as Heterophilic Cell Adhesion Molecules*

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β-Neurexins and neuroligins are plasma membrane proteins that are displayed on the neuronal cell surface. We have now investigated the interaction of neurexin 1β with neuroligin 1 to evaluate their potential to function as heterophilic cell adhesion molecules. Using detergent-solubilized neuroligins and secreted neurexin 1β-IgG fusion protein, we observed binding of these proteins to each other only in the presence of Ca2+ and in no other divalent cation tested. Only neurexin 1β lacking an insert in splice site 4 bound neuroligins, whereas neurexin 1β containing an insert was inactive. Half-maximal binding required 1–3 μM free Ca2+, which probably acts by binding to neurexin 1β but not to neuroligin 1β. To determine if neurexin 1β and neuroligin 1β can also interact with each other when present in a native membrane environment on the cell surface, we generated transfected cell lines expressing neuroligin 1 and neurexin 1β. Upon mixing different cell populations, we found that cells aggregate only if cells expressing neurexin 1β are mixed with cells expressing neuroligin 1β. Aggregation was dependent on Ca2+ and was inhibited by the addition of soluble neurexin 1β lacking an insert in splice site 4 but not by the addition of neurexin 1β containing an insert in splice site 4. We conclude that neurexin 1β and neuroligin 1 (and, by extension, other β-neurexins and neuroligins) function as heterophilic cell adhesion molecules in a Ca2+-dependent reaction that is regulated by alternative splicing of β-neurexins.

Neurons in the brain are connected to each other by thousands of synapses, creating a dense network of communicating cells. Cell recognition processes between neurons are likely to contribute to the establishment and maintenance of this network, but little is known about the mechanisms involved. Neurexins constitute a family of polymorphic cell surface proteins that are candidates for mediating cell recognition between neurons (1, 2). Three mammalian genes for neurexins are known. Each gene has two promoters that drive the synthesis of long and short classes of transcripts (1–4). The long transcripts encode α-neurexins, and the short transcripts encode β-neurexins. Both transcripts are detectable only in neurons (1, 5).

α- and β-neurexins are type I membrane proteins that resemble cell surface receptors and are composed of canonical sets of domains. The extracellular sequences of α-neurexins contain a classical N-terminal signal peptide followed by six weakly homologous repeats with interspersed EGF1-like sequences. The six repeats are related to repeated sequences found in a number of proteins and were first described in the G domain of laminin A, sex hormone-binding globulin, and neurexins (1, 6, 7). For this reason we call these repeats LNS (laminin/neurexin/sex hormone-binding globulin) domains. In α-neurexins, EGF-like sequences are placed after the first, third, and fifth LNS domain. After the sixth LNS domain, α-neurexins contain a short serine/threonine-rich sequence that is probably O-glycosylated (4). At the C terminus, α-neurexins have a single transmembrane region followed by a conserved short cytoplasmic sequence (55 amino acids).

β-Neurexins are largely identical with α-neurexins except that they lack the first five LNS domains and the three EGF-like sequences. Instead, their N terminus is composed of an atypical signal peptide that is cleaved in vivo and is followed by a short sequence specific for β-neurexins (4). The remainder of the β-neurexins is identical with the C-terminal half of the α-neurexins starting with the sixth LNS domain. The major difference between α- and β-neurexins is that α-neurexins contain five LNS and three EGF domains at the N terminus that are absent from β-neurexins. Therefore, β-neurexins are N-terminally truncated forms of α-neurexins with a short unique N-terminal sequence.

All neuroligin transcripts are subject to extensive alternative splicing that may result in thousands of isoforms (5). There are five sites of alternative splicing in α-neurexins, labeled splice sites 1–5. By contrast, β-neurexins have only two sites of alternative splicing that correspond to splice sites 4 and 5 of α-neurexins. Different neurons express distinct combinations of differentially spliced α- and β-neurexins (5). In addition to the classical neurexins, a protein in Drosophila was recently called neurexin IV whose human homologue is CASPR (8, 9). This protein has an interesting function in Drosophila development but should not be considered a neurexin, because it is only distantly homologous to vertebrate neurexins, has a different domain structure, is expressed outside of the nervous system, and is not subjected to alternative splicing. The properties of its human homologue suggest a developmental function distinct from neurexins (9).

Neuroligins represent a family of cell surface proteins that were discovered because they were stoichiometrically purified on an affinity matrix made from immobilized β-neurexins (11, 12). Similar to neurexins, neuroligins are type I membrane proteins composed of a long extracellular sequence that is glycosylated, a single transmembrane region, and a relatively short intracellular sequence. Different from neurexins, however, most of the extracellular sequences of neuroligins form a single large domain that is homologous to esterases, such as

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acetylcholine esterase (11, 12). The affinity purification of neuroligins on immobilized β-neurexins suggests that they bind to β-neurexins. Neuroligins are purified only on β-neurexins but not α-neurexins, suggesting that their interaction requires the presence of the short N-terminal sequence that is unique to β-neurexins. Furthermore, neuroligins are only purified on immobilized β-neurexins that lack an insert in splice site 4, which is located in the single LNS domain of β-neurexins. β-Neurexins containing an insert do not retain neuroligins. This result suggests that the binding of neuroligins to β-neurexins involves the LNS domain in addition to the N-terminal presence of Ca^{2+}, indicating that a structural Ca^{2+}-binding site in neuroligins or neuroligins may be involved in the interaction.

Since neuroligins only bind to a subset of β-neurexins, the other β-neurexins that are not included in this subset and all α-neurexins could potentially bind to other ligands. So far, only one additional potential ligand for neurexins has been found, a 29-kDa protein called neurexophilin that binds only to α-neurexins (13). Neurexophilin is produced in a small subset of neurons, whereas neuroligins are synthesized in all neurons. The structure of neurexophilin suggests that it is synthesized as a larger precursor protein that is proteolytically processed and secreted similarly to neuropeptides (13). Inasmuch as only two potential neurexin ligands are known, it seems likely that many additional ligands for neuroligins remain to be discovered.

The receptor-like structure of the neurexins suggests that they could in principle serve two types of function that are not mutually exclusive: a function as signaling receptors, which transduce an extracellular messenger into an intracellular response, or a function as cell adhesion molecules, which serve to mediate the binding of cells to each other. Both types of function are compatible with each other and with a general role as “recognition molecules.” The possible involvement of neuroligins in cell recognition between neurons is based on the following properties (1–5): 1) neuroligins are expressed at detectable levels only in neurons; 2) they are cell surface proteins with a receptor-like structure; 3) they are present in multiple isoforms created by the presence of three genes per genome, two different promoters per gene, extensive alternative splicing per transcript, and multiple variants for many sites of alternative splicing; and 4) different neuroligins express different combinations of alternatively spliced neuroligins.

A possible function for neuroligins as cell recognition molecules would most likely be performed by interactions with isoform-specific ligands. The two currently known neuroligin ligands, neuroligins and neurexophilin, could potentially serve the two types of recognition functions. Neurexophilin resembles a neuropeptide and might function as an extracellular messenger that binds to α-neurexins as a receptor. However, a signaling function for neurexophilin has not yet been demonstrated. Neuroligins resemble cell surface receptors similar to neuroligins and might form heterotypic junctions by binding to β-neurexins. Since β-neurexins and neuroligins are both intrinsic membrane proteins of the neuronal cell surface, they could conceivably function as cell adhesion molecules and form heterophilic junctions between neurons. However, such a function has not been investigated to date. All interactions between neuroligins and β-neurexins were performed with solubilized proteins. It is unclear if neuroligins and neuroligins can bind to each other when in a membrane and, if so, whether they mediate cell adhesion or interact on the same cell surface to form surface heterodimers.

In the present study, we have addressed the possibility that neuroligin 1β and neuroligin 1 form a heterophilic intercellular junction by functioning as cell adhesion molecules. Our study has two goals: 1) to analyze the properties of the interaction between neurexin 1β and neuroligin 1 and its dependence on Ca^{2+}, and 2) to test if the in vitro binding between β-neurexins and neuroligins translates into an in vivo interaction of cells expressing β-neurexins and neuroligins. Our data demonstrate that neurexin 1β and neuroligin 1 trigger cell adhesion in a native membrane environment. The properties of the cell adhesion reaction mirror those of in vitro binding. These results suggest that β-neurexins and neuroligins form heterophilic intercellular junctions between neurons.

MATERIALS AND METHODS

Construction of Expression Vectors and Protein Production in COS Cells—For expression of neuroligin 1 and neurexin 1β in stably transfected (s2 cells (14), inducible expression plasmids were constructed as follows. The coding region of neuroligin 1 was amplified by PCR from pCMVNL1 (11, 12) (primers CGCGATCTGGACATGACTTCCGAGATCATG and CAT-TCGAAAC6GATATGACGACGCGG). The PCR product was cut with BglII and SalI and cloned into the BamHI and SalI sites of plasmid pRMA2-L (15), resulting in pRMA2-LNL. PCR of neurexin 1β was performed on a cDNA encoding neurexin 1β without an insert in splice site 4, which carried primers GGAGGATCCACTTGACTTCCGAGATCATG and GAAGGATCCACTTGACTTCCGAGATCATG. The PCR product was cut with KpnI and BglII and cloned into KpnI and BamHI sites of pRMA2-L (15), resulting in pRMA2-LNL. The expression vector encoding the IgG fusion protein of the extracellular domain of neuroligin 1 (amino acids 1–698) was generated by cloning the PCR product obtained with pCMVNL1 as template and with the primers CGCGATCTGGACATGACTTCCGAGATCATG and CCT-GTTCGGGTGAAATGGAGATCATG. The product was cut with MluI and SalI and cloned into the same sites of pCMV5. The insert from this intermediate was then transferred into the EcoRI and SalI sites of pGEX-KG vector (16). All constructs were verified by DNA sequencing. Production of IgG fusion proteins in COS cells and purification by protein A beads was done as described (4).

Binding of Neuroligins from Rat Brain to β-Neurexin IgG Fusion Protein—Rat brains were homogenized in 20 mM Tris, pH 7.5, 0.15 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride and extracted with 1% (v/v) Triton X-100 under stirring at 4°C for 90 min. The mixture was centrifuged at 100,000 × g to remove insoluble materials. The supernatant was incubated with β-neurexin IgG fusion proteins immobilized on protein A beads at 4°C for 4–6 h. For the Ca^{2+} concentration curve, free Ca^{2+} concentrations were set with Ca^{2+}/EGTA buffers (17). For the cationic specificity studies, buffers contained 4 mM EGTA with no additions (buffer only; see Fig. 1) or 4.5 mM of the indicated divalent cations. Proteins bound to the protein A beads containing the IgG fusion proteins were eluted with sample buffer, and neuroligin binding to neurexin 1β was analyzed by immunoblotting with polyclonal antibodies to neuroligin 1 (L067) and to neurexin 1β (P182) followed by 125I-labeled secondary antibodies and exposure to film or to PhosphorImager plates for subsequent quantitation, and neuroligin binding was normalized for the amount of neurexin 1β IgG fusion protein present. Ca^{2+} blotting experiments were performed essentially as described (18). Briefly, 10 μg of purified IgG neurexin 1β-1, IgG neurexin 1β-3, IgG neurexin 1, and bovine calmodulin were electrophoresed on SDS gels and transferred to nitrocellulose membranes. The membranes were incubated in 50 mM KCl, 5 mM MgCl₂, and 10 mM Tris, pH 7.0, for 10 min at room temperature. The buffer was then exchanged with fresh buffer and 1 μCi/ml [³⁵S]Ca^{2+} (ICN Pharmaceuticals, Inc.) and incubated for 10 min at room temperature. Membranes were rinsed with distilled, deionized water for 5 min, dried at room temperature, and exposed to film overnight.

Tests of the Effect of Ca^{2+} on Recombinant Neurexin 1β Monitored by Circular Dichroism—Expression of the neurexin 1β GST fusion protein was induced in E. coli with 30 μM isopropyl-1-thio-β-D-galactopyranoside for 16 h at room temperature. The GST fusion protein was purified by affinity chromatography with glutathione-agarose beads (Sigma), and neurexin 1β was cleaved from the GST with thrombin in 20 mM
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Tris, pH 8.0, 0.15 M NaCl, and 2.5 mM CaCl₂, at room temperature for 45 min. Eluate containing neurexin 1β protein was collected on ice, diluted 5-fold with 20 mM Tris, pH 8.0, and applied onto a Mono Q HR 10/10 FPLC column (Pharmacia Biotech Inc.). Thrombin and most of the bacterial protein contaminants were retained on the Mono Q column, whereas neurexin 1β protein eluted in the flow-through and was dialyzed overnight at 4 °C with 50 mM sodium phosphate, pH 6.0, 2 mM EDTA, and 10% glycerol. Neurulin 1β was further purified on a SP Sepharose XK 16/20 column eluted by a linear gradient from 0 to 1 M NaCl in 240 ml at 4 ml/min. Fractions containing neurulin 1β were pooled and concentrated to >10 mg/ml (Amicon Centriprep). Finally, 1 ml of neurulin 1β was applied onto a 75 HiLoad 16/60 FPLC gel filtration column pre-equilibrated with 20 mM Hepes, pH 7.5, 0.15 M NaCl, and 1 mM EDTA, and eluted at 1 ml/min. The protein was then homogeneously pure. It was concentrated to 20 mg/ml, frozen in aliquots in liquid nitrogen, and stored at −80 °C.

Circular dichroism spectra were recorded in an Aviv model 62DS spectropolarimeter using a 1-mm path length cuvette with approximately 10 μg of protein in 10 mM Tris, pH 7.4, 50 mM NaCl with and without 2 mM Ca²⁺ or Mg²⁺. Thermal denaturation was monitored by changes in circular dichroism absorption at 217 nm as a function of temperature (25–95 °C in 1 °C steps). The fraction of unfolded protein at each temperature was calculated as \( I_u/I_u(1 - I_u) \) (19, 20). \( I_u \) is the observed signal intensity at the respective temperature. \( I_u \) represents the signal intensities of the unfolded and folded states, respectively. The values of \( I_u \) and \( I_u(1 - I_u) \) were generated by extrapolating the linear regions of the unfolding and folding curves.

Drosophila Cell Culture and Transfection—S2 cells were grown at 25 °C in complete Drosophila medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 0.1 g/liter streptomycin (Life Technologies, Inc.). Expression plasmids were cotransfected with the plasmid pPC4 (21) to confer a selectable marker. One day after cells were plated (1–2 × 10⁶ cells/25-cm² flask in 4 ml of complete medium), 2 × 10⁶ cells/ml were resuspended in balanced saline solution (55 mM NaCl, 0.15M NaCl, and 2.5 mM CaCl₂) with cellular aggregates counting as the selectable marker. One day after cells were plated (1–2 × 10⁶ cells/25-cm² flask in 4 ml of complete medium), the DNA/liposome mixture was gently overlaid onto the cells. The cells were washed once between the liquid and the lid. The cell mixtures were then incubated at 25 °C, and then the transfection medium was replaced with complete growth medium. After 48 h to allow for the expression of drug resistance, 5 mg/liter α-amanitin was added to the cells in complete medium. Massive cell death occurred after 1–2 weeks. Cells were then removed from the flask, pelleted, and resuspended in fresh complete medium with 5 mg/liter α-amanitin. Within 2 weeks, viable cells continued to grow to high density, and thereafter cells were diluted 1:3 in fresh complete medium with α-amanitin about every week. The selection with α-amanitin was continued for three passages. Cell cloning was performed by diluting cells to 20, 40, and 80 cells/ml in 50% conditioned growth medium and aliquoted in 0.1 ml into 96-well plates with one plate per cell concentration. After 3 weeks, the plates could be seen with the unaided eye. They were split into two wells, and one was used for screening by Western blots with antibodies to neurulin (L067) and neurulin (A473). Positive clones were further analyzed for clonal homogeneity by immunocytochemistry.

Cell Aggregation Assays—Assays were modified from Refs. 22–24. Protein expression in transfected cells was induced by adding 0.7 mM CuSO₄ to the culture medium 16–20 h before the experiments. Cells were collected by centrifugation, washed once with serum-free medium, and resuspended by pipetting in balanced saline solution (55 mM NaCl, 40 mM KCl, 15 mM MgSO₄, 10 mM CaCl₂, 20 mM glucose, 50 mM sucrose, 2 mg/ml bovine serum albumin, and 20 mM Tricine, pH 6.9). Cells were counted with a hemocytometer and diluted in balanced saline solution so that the final cell density in each aggregation assay was approximately 5 × 10⁶ cells/ml. Cell suspensions (0.33-ml total volume) were placed in 0.5-ml polypyrrole test tubes, leaving a small air bubble between the liquid and the lid. The cell mixtures were then incubated at 4 °C under gentle agitation. The extent of cell/cell aggregation was measured at the indicated times by removing aliquots, spotting them onto slides, and counting the cells with a hemocytometer. Cell aggregation was calculated from the ratio \( N_c/N_u \), where \( N_c \) is the total number of cellular particles at the incubation time 0, and \( N_u \) is the total number of cellular particles at incubation time \( t \), with cellular aggregates counting as single particles. In the experiments examining the Ca²⁺ dependence of cell aggregation, cells were resuspended in balanced saline solution without Ca²⁺ containing either 2 mM EGTA or 2 mM MgCl₂.

Inhibition experiments of cell aggregation were performed by preincubating neurulin 1 cells with 1 μM of recombinant IgG fusion proteins at 4 °C for 1 h. Neurulin 1β cell clones were then added, and the incubation was continued for 90 min at 4 °C with agitation. Because of the high expression of neurulin 1 and neurulin 1β and the cooperative nature of cell aggregation, the number of cells used was reduced to 1 × 10⁶ cells/ml, and the CuSO₄ induction time was reduced to 8–10 h.

RESULTS

Divalent Cation Specificity of the Interaction between Neurulin 1β and Neurexin 1β—Previous studies showed that neurulins bind to immobilized β-neurexins under two conditions: 1) Ca²⁺ had to be added to the buffer; and 2) the β-neurexins used had to lack an insert in splice site 4 (11, 12). The first finding suggested the possibility that Ca²⁺ binds to β-neurexins or neurulins or both and that Ca²⁺ binding enables these molecules to interact with each other. The second finding indicated that the alternative splicing of β-neurexins regulates binding. To gain further insight into how neurulins and neurulins bind to each other, we have now studied the effects of different divalent cations on their interactions.

We used immobilized IgG neurulin 1β fusion proteins without (IGN Iβ-1) or with an insert in splice site 4 (IGN Iβ-3) as affinity matrices for rat brain proteins. Detergent-solubilized rat brain proteins were applied to the columns containing immobilized neurulin 1β in the presence of different divalent cations. We found that neurulins were retained on the column only in the presence of Ca²⁺, whereas closely related divalent cations, Ba²⁺ and Sr²⁺, were unable to substitute for Ca²⁺ at a 0.5 mM free ion concentration (Fig. 1). Furthermore, neurulin binding was specific to neurulin 1β lacking an insert in splice site 4 as reported before, and other divalent cations were not able to overcome this binding specificity. Thus, Ca²⁺ action on the binding of neurulin 1β to neurulins is highly specific and cannot be substituted for by other cations, suggesting that a specific Ca²⁺-binding site is involved in binding of neurulins to neurulin 1β.

Ca²⁺ Concentration Dependence of the Interaction of Neurulins with Neurexin 1β—To determine the apparent affinity of the Ca²⁺ binding site that is required for binding neurulins to neurulin 1β, we performed the affinity chromatography experiments as described above at different concentrations of free Ca²⁺. Concentrations of free Ca²⁺ were set with Ca²⁺/EGTA buffers (17). Neurulin binding to neurulin 1β was activated at very low concentrations of Ca²⁺ (Fig. 2a). We quantitated the binding reaction as a function of free Ca²⁺ by measuring the immunoblotting signal radioactively and by normalizing the
Neuroligins or neuroligins? To address this question, we analyzed Ca²⁺ binding to recombinant extracellular domains of neurexin 1β and neuroligin produced as IgG fusion proteins. Although the neurexin 1β-IgG fusion proteins expressed well, the neuroligin IgG fusion protein was produced only in low amounts, maybe because of its large size and high number of disulfide bonds. Therefore, our choice of Ca²⁺-binding assays was limited, and we used ⁴⁵Ca²⁺ blotting as a method because it requires relatively little protein and can be performed simultaneously for all proteins (18). No ⁴⁵Ca²⁺ signal was detected for neurexin 1β with or without an insert in splice site 4. In contrast, equivalent amounts of neuroligin 1 protein, corresponding to fewer molecules because neuroligin is larger, bound ⁴⁵Ca²⁺ on the blots, albeit weakly (Fig. 3). Calmodulin, used as a positive control, exhibited a much stronger signal. The stronger calmodulin signal may be due to the 4-fold difference in the number of molecules of calmodulin versus IgG neuroligin 1 loaded onto the gel and the higher Ca²⁺ affinity of calmodulin. Nevertheless, the signal obtained with neuroligin 1 suggests that it binds Ca²⁺ and may be responsible for the Ca²⁺ dependence of the neurexin 1β/neuroligin interaction.

**Fig. 2.** Ca²⁺ concentration dependence of neuroligin 1 binding to neurexin 1β. a, Ca²⁺ titration of the binding of neuroligin 1 to immobilized neurexin 1β IgG fusion protein (IGN 1β-1) in a representative experiment. Immobilized IGN 1β-1 was used as an affinity matrix for neuroligins from rat brain homogenates in the presence of the indicated concentrations of free Ca²⁺. Bound neuroligin 1 was analyzed by immunoblotting (anti-NL). To control for differences in the amount of affinity matrix, the neurexin 1β fusion protein was visualized in the same blot with an antibody to neurexin 1β (anti-N 1β). Coomassie Blue staining revealed similar patterns of bound proteins as described previously (11, 12), with neuroligin as the only specific binding protein and the major protein component of the affinity eluates (not shown). Numbers on the left indicate positions of molecular weight markers. b, quantitation of multiple experiments similar to those described in a. Immobilized neurexin 1β IgG fusion proteins lacking (IGN 1β-1) or containing (IGN 1β-3) an insert in splice site 4 were used for affinity chromatography of neuroligins from brain homogenates. Binding of neuroligin 1 was quantified using immunoblots with iodinated secondary antibodies and PhosphorImager detection. Error bars represent means ± S.E. from duplicates of two experiments.

**Fig. 3.** Ca²⁺ binding to calmodulin, β-neurexins, and neuroligin 1 analyzed by ⁴⁵Ca²⁺ blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by blotting with ⁴⁵Ca²⁺ (left panel) or by Coomassie Blue staining (right panel). ⁴⁵Ca²⁺ blotting fails to reveal a signal with IgG fusion proteins of neurexins 1β without or with an insert in splice site 4 (IGN 1β-1 and -3, respectively) even after prolonged PhosphorImager exposure. Neuroligin 1 IgG fusion protein (IGNL1) exhibited much weaker binding than calmodulin, possibly because calmodulin remains in a more native state after SDS treatment. Positions of the four proteins analyzed are indicated by arrows. Numbers on the left indicate positions of molecular weight markers.

Signal for the amount of IgG fusion protein present in the assay. Quantitation showed that half-maximal binding of neurexin 1β to neuroligins required approximately 2 μM free Ca²⁺ (Fig. 2b). As before, only neurexin 1β lacking an insert in splice site 4 bound, whereas neurexin 1β containing an insert was inactive. Our data suggest that the interaction of neurexin 1β with neuroligins is activated by one or several high affinity Ca²⁺-binding sites. Since extracellular Ca²⁺ concentrations are millimolar, these binding sites are presumably saturated in vivo, suggesting that Ca²⁺ is a structural component of β-neurexins or neuroligins.

**⁴⁵Ca²⁺ Blotting to Neurexins 1β and Neuroligin 1**—Our data suggest that a structural Ca²⁺ binding site in β-neurexin or neuroligins or both needs to be occupied in order for the proteins to interact. Which of these two proteins contains the Ca²⁺-binding site, or do both of them contain Ca²⁺-binding sites? To address this question, we analyzed Ca²⁺ binding to the recombinant extracellular domains of neurexin 1β and neuroligin 1. As expected from its ability to interact with neuroligin 1, however, no change in the circular dichroism spectrum was observed as a function of Ca²⁺, indicating that Ca²⁺ did not bind (data not shown). We next used the more sensitive method of temperature denaturation, in which the denaturation of the protein by increasing
the temperature as a function of Ca$^{2+}$ by circular dichroism at a conformation-dependent wavelength. The denaturation curve monitors a protein’s conformation from the folded or native to the unfolded or denatured state as a function of temperature. Because the binding of a small molecule to a protein often stabilizes its structure, the denaturation curve often shifts to the right after Ca$^{2+}$ binding. This procedure has been successfully used to measure Ca$^{2+}$ binding to the C$_2$ domains of synaptotagmins, well characterized Ca$^{2+}$-binding proteins of synaptic vesicles (19, 20).

The denaturation temperature experiments of neurexin 1$\beta$ generated cooperative denaturation curves, confirming the conclusion that the bacterial neurexin 1$\beta$ was properly folded before the temperature increase. However, no Ca$^{2+}$-dependent changes were observed (Fig. 4). These data corroborate the $^{45}$Ca$^{2+}$ binding finding that neurexin 1$\beta$ does not bind Ca$^{2+}$, suggesting that neurexin is the likely Ca$^{2+}$-binding protein for the neuroligin/β-neurexin interaction.

Expression of Neurexin 1$\beta$ and Neuroligin 1 in Schneider S2 Cells—Neurexin 1$\beta$ and neuroligin 1 are intrinsic membrane proteins of the neuronal cell surface that bind to each. Thus, the potential exists that they might function as heterophilic cell adhesion molecules or that they interact with each other while co-localized on the same cell surface. Such a cell adhesion function would be very interesting in view of the assymmetric junction it would create and its regulation by alternative splicing. Therefore, we initiated experiments to test such a function.

We transfected Drosophila S2 cells separately with plasmids encoding either neurexin 1$\beta$ without an insert in splice site 4 or neuroligin 1. Drosophila S2 cells are nonadherent cells that facilitate cell adhesion assays (14, 22–24). Stable cell lines were generated. Transcription from the transfected plasmids is regulated by copper, allowing us to induce expression of neuroligin 1 and neurexin 1$\beta$ by the addition of CuSO$_4$ to the culture medium. Analysis of the cell lines by immunoblotting shows that high levels of protein expression are induced by CuSO$_4$ (Fig. 5). The fuzzy appearance of the protein bands suggests that neurexin 1$\beta$ and neuroligin 1 are glycosylated in the S2 cells similar to other cells. Immunofluorescence on the clonal cells showed that they expressed neuroligin 1 and neurexin 1$\beta$ partially on the cell surface (data not shown).

Heterophilic Cell Adhesion Mediated by Neuroligin 1 and Neurexin 1$\beta$—We next investigated if expression of neurexin 1$\beta$ or neuroligin 1 induced cell adhesion. Populations of neuroligin 1 cells, neurexin 1$\beta$ cells, or α-amanitin-resistant control cells incubated separately exhibited no cell adhesion in Ca$^{2+}$-containing media (Fig. 6, A, B, and H). However, when we mixed neurexin 1$\beta$ and neuroligin 1-expressing cells together, large clumps of cell aggregates were observed (Fig. 6C). These clumps were not observed when we combined the neuroligin 1 or neurexin 1$\beta$ expressing cells with control cells (F and G). Furthermore, similar to the in vitro binding reactions (Fig. 1), cell adhesion was not observed when we mixed the neurexin 1$\beta$- and neuroligin 1-expressing cells in medium containing either EGTA without divalent cations (Fig. 6D) or with Mg$^{2+}$ (Fig. 6E). These data show that neurexin 1$\beta$ and neuroligin 1 function as heterophilic cell adhesion molecules. Since cells expressing only neurexin 1$\beta$ or neuroligin 1 do not aggregate, these proteins are not homophilic cell adhesion molecules. The similarity in cation requirement between the cell adhesion assays (Fig. 6) and the in vitro binding experiments (Fig. 1) indicates that the binding characterized with detergent-solubilized proteins forms the basis for the cell adhesion activity of neurexin 1$\beta$ and neuroligin.

Time Course of Cell Adhesion Mediated by Neurexin 1$\beta$ and Neuroligin 1—We quantified cell/cell adhesion by analyzing aliquots of the cell aggregation reaction with a hemocytometer at different reaction times and counting the number of particles. The quantitative analysis confirms the qualitative finding from Fig. 6 that only mixtures of cells expressing neurexin 1$\beta$ and neuroligin 1 are capable of cell adhesion (Fig. 7). The analysis also shows that Ca$^{2+}$ is required as predicted from the in vitro binding experiments.

Next we studied the time course of cell adhesion (Fig. 7). A lag period of approximately 20–30 min was required before we observed cell adhesion. Thereafter, cell adhesion was rapid, with completion reached in approximately 60 min. The kinetics...
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and extent of cell aggregation that we observed for neurexin 1β- and neuroligin 1-expressing cells were similar to those of cells expressing other cell adhesion molecules such as the fasciclin and cadherins (24–26). Although fasciclin and cadherins are homophilic cell adhesion molecules whereas neurexin 1β and neuroligin 1 are heterophilic, the similarity of their cell adhesion properties supports a physiological role.

Soluble Neurexin 1β Inhibits Cell Adhesion—Heterotypic cell adhesion between cells expressing neurexin 1β and neuroligin 1 is dependent on Ca2+, similar to the binding of neurexin 1β to neuroligin 1 in vitro. To directly test if binding of neurexin 1β to neuroligin 1 forms the basis for the cell adhesion observed with the transfected cells, we investigated the ability of exogenous neurexin 1β to inhibit cell adhesion between transfected cells. We produced three IgG fusion proteins: neurexin 1β without an insert in splice site 4 (IGN Iβ-1), neurexin 1β with an insert in this splice site (IGN Iβ-3), and an IgG fusion protein containing only a signal sequence followed by short peptide sequence as a negative control (control IG) (4). The neurexin 1β-IgG fusion proteins were used to analyze the in vitro neurexin 1β/neuroligin 1 binding reaction (Figs. 1 and 2).

When we added the recombinant proteins to the aggregation reaction at 1 μM final concentration, only the neurexin 1β protein lacking an insert in splice site 4 inhibited (Fig. 8a). Quantitation of the reaction confirmed the specificity (Fig. 8b). These data show that in the cell adhesion assay, only one splice variant of neurexin 1β was active, similar to the in vitro binding reaction. This result suggests that the in vitro binding reaction reflects the basis of the cell adhesion function of neurexin 1β and neuroligin 1 and supports the hypothesis that alternative splicing regulates cell adhesion mediated by β-neurexins and neuroligins.

DISCUSSION

Neurexins represent a family of polymorphic cell surface proteins with receptor-like structures (1–4). We have proposed that neurexins might function as cell recognition molecules in mediating cell/cell interactions in the nervous system. This proposal was prompted by the observation of large numbers of neurexin isoforms, of their preferential expression in neurons, and of the regulated expression of distinct combinations of neurexins in different neurons (5). A function for neurexins as cell recognition molecules would require binding of neurexins to ligands that are specific for subsets of neurexins and whose binding to neurexins mediates interactions between neurons. With neuroligins, we previously identified candidate ligands for neurexins for such a function based on the following properties (11, 12): 1) neurexins and neuroligins represent cell surface proteins resembling receptors; 2) both proteins are expressed at detectable levels only in neurons; 3) neuroligins only bind to an alternatively spliced subset of β-neurexin in vitro; 4) the alternative splicing of β-neurexins that specifies their binding to neuroligins is spatially regulated in brain. The binding properties and structures of β-neurexins and neuroligins raise the possibility that they mediate heterophilic cell adhesion between neurons. However, previous studies were only performed with solubilized protein, making it uncertain if the in vitro binding between β-neurexins and neuroligins translates into an in vivo interaction between cells expressing them. We have now tested the ability of neurexin 1β and neuroligin 1 to mediate cell adhesion.

Our data show that neurexin 1β and neuroligin 1 mediate cell adhesion between cells in a Ca2+-dependent manner. No other divergent cation tested substitutes. Cell adhesion depends on the presence of both proteins. It is inhibited by the addition
FIG. 8. Inhibition of Ca\(^{2+}\)-dependent aggregation of neurexin 1\(\beta\) cells with neuroligin 1 cells by excess soluble neurexin 1\(\beta\). a, equal mixtures of neurexin 1\(\beta\)- and neuroligin 1-expressing cells were mixed as described in Fig. 6 for 90 min in the presence of Ca\(^{2+}\) without additions (part A) and with additions of excess IgG fusion proteins of neurexin 1\(\beta\) without an insert in splice site 4 (IGN 1\(\beta\); part B) or with an insert in splice site 4 (IGN 1\(\beta\); part C). In part D, control IgG fusion protein containing only a short N-terminal peptide fused to IgG was added. The arrows point to cell aggregates. b, quantitation of experiments similar to those shown in a. Aggregation is expressed as the number of free cells at the beginning of plating (N\(_0\)) minus the number of free cells after 90 min of incubation (N\(_{\text{free, min}}\)) divided by N\(_0\). The graph shows means ± S.E. from four determinations.

of soluble truncated neurexin 1\(\beta\) if the added neurexin 1\(\beta\) lacks an insert in splice site 4 but not if it contains an insert in splice site 4. Thus, the characteristics of cell adhesion mirror the properties of in vitro binding between neurexin 1\(\beta\) and neuroligin 1. This binding is also Ca\(^{2+}\)-activated and is also dependent on alternative splicing. Ca\(^{2+}\) acts by binding to a high affinity site, since only 1–3 \(\mu\)M free Ca\(^{2+}\) are required for half-maximal activation of the interaction. Ca\(^{2+}\) probably binds to neuroligin 1, since the \(^{45}\)Ca\(^{2+}\) blot detected only binding to neuroligin 1 but not neurexin 1\(\beta\), and since recombinant neurexin 1\(\beta\) exhibited no Ca\(^{2+}\) binding in other, more native and sensitive assays. Thus, neurexin 1\(\beta\) and neuroligin 1 function as heterophilic cell adhesion molecules. Since neurexins 2\(\beta\) and 3\(\beta\) and neuroligins 2 and 3 have binding properties similar to those of neurexin 1\(\beta\) and neuroligin 1 (11, 12), it is likely that other neurexins and neuroligins are also cell adhesion molecules.

Our data establish a function for \(\beta\)-neurexins and neuroligins in mediating heterophilic cell adhesion. Based on the cell aggregation assays, the time course and strength of cell adhesion mediated by the interaction between \(\beta\)-neurexins and neuroligins appear to be similar to other cell adhesion reactions, e.g. those observed with cadherins and fasciculins (24–26). However, the latter molecules mediate homophilic cell adhesion, whereas \(\beta\)-neurexins and neuroligins cause heterophilic cell adhesion. In neurexin 1\(\beta\), the short unique N-terminal sequence that is specific for \(\beta\)-neurexins and the alternatively spliced LNS domain is required for binding neuroligins. LNS domains are found in many proteins that interact with receptor sites, e.g. agrin, laminin A, and slit (reviewed in Ref. 1). In neuroligin 1, the esterase-like domain is involved in binding \(\beta\)-neurexins. Similar esterase-like domains have been found in neurotactin and gliotactin, Drosophila proteins that are involved in cell/cell interactions (27, 28). The interacting partners for neurotactin and gliotactin have not yet been identified, however, and it will be interesting to discover if they also contain LNS domains.

Cell adhesion forms the basis for intercellular junctions. A function for neurexins and neuroligins in intercellular junctions is supported by the observation that neurexins intracellularly bind a molecule called CASK (29). The structure of CASK resembles PSD-95 and ZO-1, proteins that are localized to the intracellular side of intercellular junctions. It is therefore possible that binding of \(\beta\)-neurexins and neuroligins forms the nucleus for an intercellular junction. Such a putative junction has attractive features in terms of nervous system function. The heterophilic nature of their interaction would create an asymmetric junction in which the two cells on both sides of the junction are not equivalent. The regulation of the binding of \(\beta\)-neurexins to neuroligins by alternative splicing would provide a fine tuning of which neurons interact.

The formation of cell adhesion complexes between neurons by \(\beta\)-neurexins and neuroligins raises several questions. First, only a subset of neurexins bind neuroligins and are therefore involved in establishing a neurexin-neuroligin intercellular junction. What do the other neurexins do? Answers to this question will be interesting and will require identification of additional ligands, one of which may be neurexophilin. Second, do neuroligins only have a single class of ligands (i.e. neurexins), or are there other ligands for neuroligins? In terms of size, neuroligins are clearly large enough to accommodate additional binding sites. Third, where is the junction formed by \(\beta\)-neurexins and neuroligins? Experiments to address these questions are under way.

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