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Neuroligin 1: A Splice Site-Specific Ligand for β-Neurexins

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Summary

Neurexins are neuronal cell surface proteins with hundreds of isoforms generated by alternative splicing. Here we describe neuroligin 1, a neuronal cell surface protein that is enriched in synaptic plasma membranes and acts as a splice site-specific ligand for β-neurexins. Neuroligin 1 binds to β-neurexins only if they lack an insert in the alternatively spliced sequence of the G domain, but not if they contain an insert. The extracellular sequence of neuroligin 1 is composed of a catalytically inactive esterase domain homologous to acetylcholinesterase. In situ hybridization reveals that alternative splicing of neurexins at the site recognized by neuroligin 1 is highly regulated. These findings support a model whereby alternative splicing of neurexins creates a family of cell surface receptors that confers interactive specificity onto their resident neurons.

Introduction

Neurexins are polymorphic cell surface proteins expressed in neurons. They were discovered in the course of cloning the presynaptic receptor for α-latrotoxin (Ushkaryov et al., 1992). Three genes encoding neurexins are known (named 1, 2, and 3). Each neurexin gene has two independent promoters and generates two classes of mRNAs. The longer mRNAs encode α-neurexins and the shorter mRNAs β-neurexins. This results in the synthesis of six principal neurexin isoforms, called neurexins Iα to IIIβ, of which neurexin Iα corresponds to the high molecular weight component of the α-latrotoxin receptor (Ushkaryov et al., 1992, 1994).

Neurexins are expressed at significant levels only in brain. The six isoforms are coexpressed in neurons and are distributed differentially in various brain regions. Groups of neurons exhibit different combinations of neurexins, resulting in a combinatorial specificity of neurexin expression that is generated by their cell type–specific transcription from six promoters in three genes (Ullrich et al., 1995). Another remarkable characteristic of neurexins is their evolutionarily conserved pattern of extensive alternative splicing. α-Neurexins share five canonical sites of alternative splicing, the last two of which are also present in β-neurexins. At most of these splice sites, two variants are observed (negative and positive inserts). However, some of the splice sites may have more than 10 variants. As a result, the total number of neurexins in brain probably exceeds 2000 forms (Ullrich et al., 1995). The variable sequences of the neurexins render them among the most polymorphic proteins known.

The structures of the neurexins suggest a possible function as cell surface receptors. Neurexins contain epidermal growth factor–like sequences and domains homologous to the G domain repeats of laminin A, indicating a function in cell–cell interactions. A 29 kDa protein copurifying with neurexin Iα in the α-latrotoxin receptor has a structure suggestive of a soluble ligand for neurexins (A. G. Petrenko and T. C. S., unpublished data). Putative ligands for neurexins could interact with all neurexins or with subsets of neurexins characterized by a specific pattern of alternative splicing. In the latter instance, alternative splicing would regulate the ligand specificity of each neurexin, implying the existence of a large number of different ligands.

Here we report the identification of a novel neuronal cell surface protein that binds to all three β-neurexins but not to α-neurexins. The protein, called neuroligin 1 in anticipation of additional ligands for neurexins, binds only to β-neurexins that lack an insert in the alternatively spliced sequence of the G domain, but not to β-neurexins containing an insert. In situ hybridization reveals that the alternative splicing of neurexins in the G domain is regulated in a region-specific fashion. These observations suggest that different splice versions of neurexins have different ligands and support a role for neurexins in neuronal specificity.

Results

Purification of Neuroligin 1

To produce sufficient amounts of neurexins for biochemical studies, we constructed a baculovirus vector encoding a secreted form of neurexin 1β. The protein was truncated in the middle of the O-linked sugar region before the transmembrane region and fused to a His_6 sequence to facilitate purification. Recombinant neurexin 1β was produced in Sf9 cells and purified to apparent homogeneity by a series of chromatography steps (see Experimental Procedures). Purified neurexin 1β was coupled to cyanogen bromide–activated Sepharose and used as an affinity matrix for total rat brain proteins solubilized in 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) (Figure 1).

Brain proteins were loaded onto the affinity column in the presence of Ca^{2+} at physiological salt concentrations and eluted with 1 M NaCl first in the presence of Ca^{2+} (Figure 1, lanes 5–8) and then in the presence of EDTA (lanes 9 and 10). No specific binding protein was detected in the eluate containing Ca^{2+}. Elution with EDTA resulted in the purification of a single major protein of approximately 116 kDa, which we named neuroligin 1 (asterisk in Figure 1). As a control, a column containing bovine serum

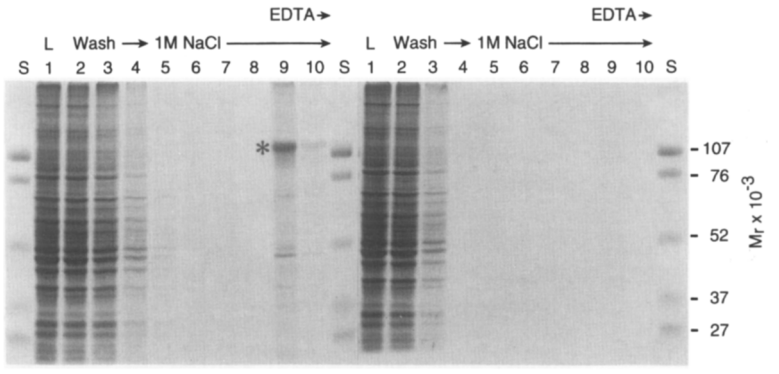

Figure 1. Purification of Neuroligin 1 by Affinity Chromatography on Immobilized Neurexin 1β

Neurexin 1β and BSA were covalently coupled to cyanogen bromide–activated Sepharose. Total rat brain proteins solubilized in CHAPS (lane 1; L, loading material) were applied to the columns in the presence of Ca^{2+}, washed with loading buffer (lanes 2–4), and eluted first with 1 M NaCl and Ca^{2+} (lanes 5–8) and then with 1 M NaCl and EDTA (lanes 9 and 10). Equivalent amounts of all fractions were analyzed by SDS–PAGE and Coomassie blue staining. Neuroligin 1 eluted from the neurexin 1β column by EDTA is marked by an asterisk. Numbers on the right indicate the size of molecular weight markers run in lanes labeled S.

albumin (BSA) showed no bound protein (Figure 1, right). Furthermore, application of liver proteins to the neurexin 1β column also failed to uncover specific binding proteins (data not shown). These experiments reveal the presence of a neurexin-binding protein in brain that tightly associates with neurexin 1β in the presence of Ca^{2+} and is purified from total brain in a single step by affinity chromatography.

Structure of Neuroligin 1

The brain protein eluted from immobilized neurexin 1β was subjected to amino acid sequencing. cDNAs encoding neuroligin 1 were cloned based on the amino acid sequences obtained (Figure 2A). An initiator methionine codon was identified in the nucleotide sequence as the first ATG that is not closely followed by a stop codon. Translation of the nucleotide sequence predicts synthesis of an 843 amino acid protein. Sequence analysis using hydrophobicity plots (Kyte and Doolittle, 1982) reveals two regions of high hydrophobicity: a short region at the N-terminus and a 22 amino acid sequence close to the C-terminus. The N-terminal hydrophobic sequence has the characteristics of a signal peptide (von Heijne, 1986) with a predicted cleavage site after residue 44. The C-terminal hydrophobic sequence has the size and hydrophobicity of a transmembrane region.

Examination of multiple cDNA clones showed that two sequences of 27 bp and 60 bp are either present or absent, respectively (Figure 2A). Because these variations were found in multiple clones, it is likely that they represent alternative splicing events. Thus, neuroligin 1, like the neurexins, is subject to alternative splicing.

All of the peptide sequences obtained from purified neuroligin 1 were found in the translated cDNA sequence, suggesting that the cDNA encodes the protein purified. Purified neuroligin 1 migrates on SDS–polyacrylamide gels as a protein of approximately 116 kDa, whereas the cDNA sequence predicts synthesis of a 95 kDa protein. The fuzzy appearance of purified neuroligin 1 on SDS gels (see Figure 1) suggests that it may be glycosylated. The structure of neuroligin 1 contains multiple scattered consensus sequences for N-linked glycosylation. In addition, a cluster of serine and threonine residues that may serve as O-linked sugar acceptor sites is present N-terminal to the transmembrane region (Figure 2A). To test whether neuroligin 1 is N-glycosylated, O-glycosylated, or both, we digested it with a series of glycohydrolases (neuraminidase, N-glycosidase F, and O-glycanase) and analyzed its size by immunoblotting. These experiments demonstrated that neuroligin 1 contains both N-linked and O-linked sugars, with the sugar residues accounting for the size difference between native neuroligin 1 and its primary translation product (data not shown). The O-glycosylation of neuroligin 1 may create a carbohydrate-rich domain just outside its transmembrane region, similar to O-linked glycosylation cassettes in other cell surface receptors.

The presence of a signal sequence and of a single transmembrane region in neuroligin 1 suggests that neuroligin 1 is a type 1 membrane protein with an extracellular N-terminus and an intracellular C-terminus. A domain model for neuroligin 1 is proposed in Figure 2B based on this transmembrane topology. According to this model, neuroligin 1 contains an extracellular sequence of 651 amino acids (after signal peptide cleavage and including both alternatively spliced sequences) and a cytoplasmic region of 126 amino acids. The domain model of neuroligin 1 is supported by the observed glycosylation of neuroligin 1 and the presence of an O-glycosylation cassette N-terminal to the membrane-spanning region. In addition, a cluster of positively charged amino acids flanks the transmembrane region on the intracellular side, as is typical for stop–transfer sequences (von Heijne and Gavel, 1988). Finally, the N-terminal esterase homology domain of neuroligin 1 (see below) further supports the assignment of the N-terminus to the extracellular space.

Neuroligin 1 Has an Esterase-like Domain

Data base searches revealed a significant homology of almost the entire extracellular domain of neuroligin 1 with members of the esterase protein family. This diverse family of proteins includes true esterases such as acetylcholinesterase and carboxylesterase, as well as nonesterase proteins such as thyroglobulin and neurotactin (de la Escalera et al., 1990; Hortsch et al., 1990; Takagi et al., 1988). An alignment of the neuroligin 1 sequence with sequences of selected esterase domain proteins shows that the homology of neuroligin 1 to other members of this

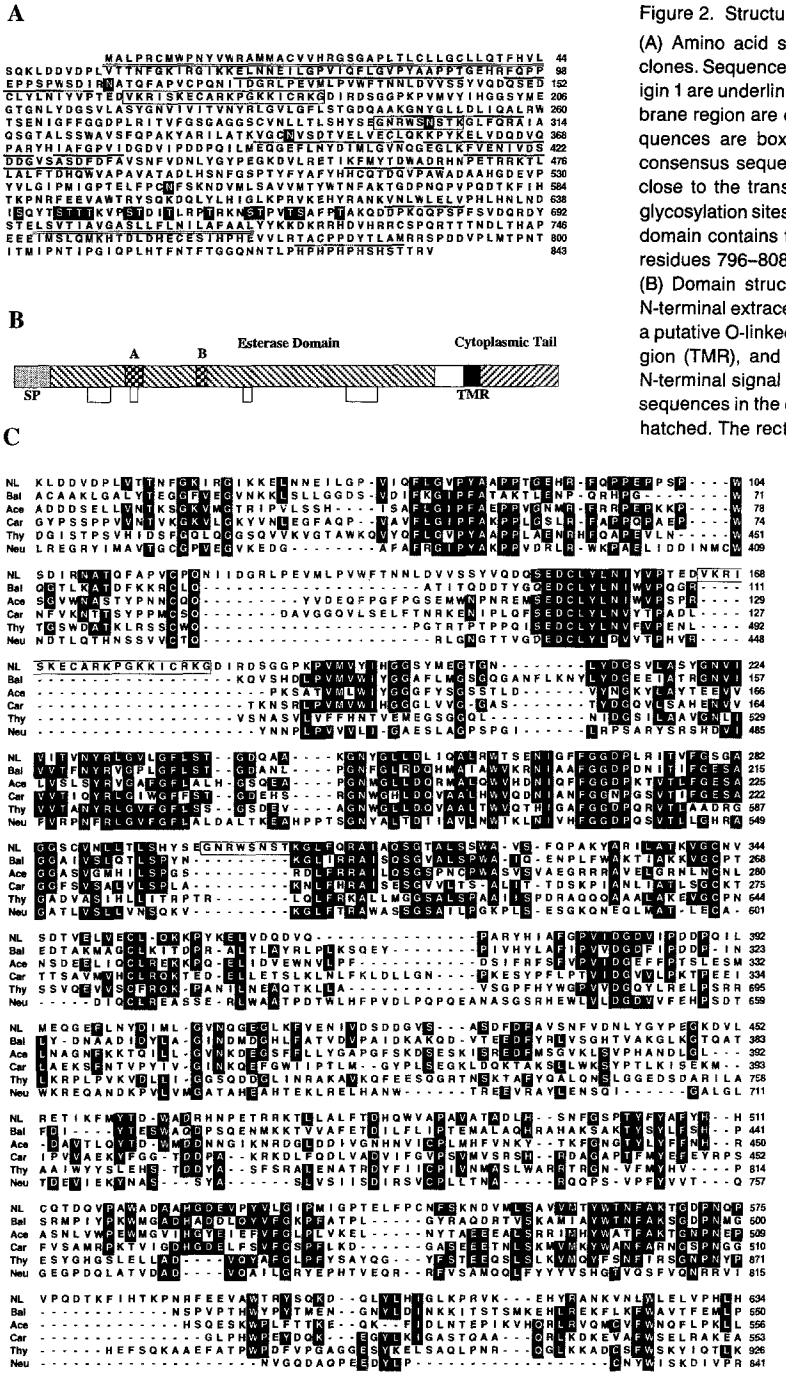

Figure 2. Structure of Neuroligin 1

(A) Amino acid sequence of rat neuroligin 1 deduced from cDNA clones. Sequences determined of tryptic peptides from purified neuroligin 1 are underlined, and the putative signal sequence and transmembrane region are double underlined. The two alternatively spliced sequences are boxed. Asparagine resides of N-linked glycosylation consensus sequences and clustered serine and threonine residues close to the transmembrane region that serve as putative O-linked glycosylation sites are shown on a black background. The cytoplasmic domain contains two copies of a 6-residue repeat (MTPNTI/MIPNTI; residues 796–808).

(B) Domain structure of neuroligin 1. Neuroligin 1 is composed of N-terminal extracellular domains that include an esterase domain and a putative O-linked glycosylation domain, a single transmembrane region (TMR), and a C-terminal cytoplasmic sequence. The putative N-terminal signal peptide is labeled SP. The two alternatively spliced sequences in the esterase domain are labeled A and B and are cross-hatched. The rectangular lines below the bar denote proposed disulfide loops based on the crystal structure of acetylcholinesterase (Sussman et al., 1991).

(C) Sequence alignment of neuroligin 1 with five representative members of the esterase domain family. Sequences are identified on the left and numbered on the right. Abbreviations: NL, neuroligin; Bal, rat bile acid–activated esterase (Han et al., 1987); Ace, Torpedo acetylcholinesterase (Sikorac et al., 1987); Car, rat carboxylesterase ES-10 (Robbi et al., 1990); Thy, rat thyroglobulin (Di Lauro et al., 1985); Neu, Drosophila neurotactin (de la Escalera et al., 1990; Hortsch et al., 1990). Residues identical in at least 50% of the sequences are shown on a black background. Hyphens indicate spaces. Note that the active site serine (corresponding to residue 281 in neuroligin 1) is substituted for an inactive residue in neuroligin 1, neurotactin, and thyroglobulin. Alternatively spliced segments of neuroligin 1 are boxed.

family is uniformly distributed over the entire length of the domain (Figure 2C). As it is in thyroglobulin and neurotactin, the serine residue at the active site in neuroligin 1 is replaced by an inert amino acid, suggesting that neuroligin 1 is not a catalytically active esterase. Of the six disulfide-bonded cysteine residues in acetylcholinesterase, four are conserved in all members and allow a tentative assignment of disulfide bonds in neuroligin 1 (Figure 2B). The alternatively spliced segments of neuroligin 1 map to loops in the crystal structure of acetylcholinesterase (Sussman et al., 1991), indicating that these sequences are accommodated in the tertiary structure of neuroligin 1 as extended surface loops. Interestingly, the longer alternatively spliced sequence contains two cysteines and may form a disulfide-bonded ring (Figure 2B).

A quantitative assessment of the degree of relatedness of neuroligin 1 to other members of the esterase domain family reveals that it is similarly related to all members (32%–36% sequence identity), with small differences among proteins. Neurotactin is the only member of the

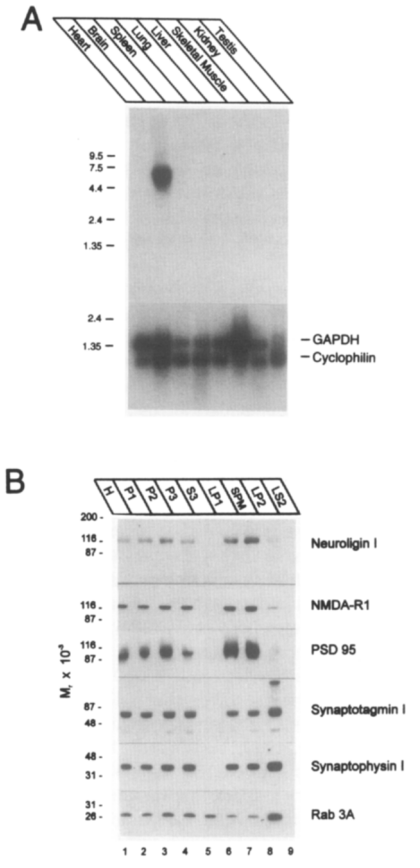

Figure 3. Brain-Specific Expression and Subcellular Localization of Neuroligin 1

(A) Tissue distribution of neuroligin 1 expression. A blot containing total RNA from the indicated rat tissues was hybridized with a uniformly labeled neuroligin 1 probe and exposed to film at −70°C for 1 hr (top). The same blot was rehybridized with GAPDH and cyclophilin probes to control for RNA loads (bottom). Numbers on the left indicated positions of molecular weight markers (in kilobases).

(B) Subcellular fractionation of brain. Crude synaptosomes were isolated by differential centrifugation (P2), lysed hypoosmotically, and separated into fractions enriched in heavy membranes (LP1), in SPMs, in synaptic vesicles (LP2), and in cytosol (LS2). Equal aliquots of each fraction were immunoblotted using antibodies against the indicated proteins. Numbers on the left indicate positions of molecular weight markers (in kilobases).

esterase family currently listed in the data bases that, in addition to neuroligin 1, contains a transmembrane region, but neuroligin is less related to neurotactin (27% identity) than to other esterase proteins. Moreover, neurotactin is a type 2 membrane protein with an N-terminal transmembrane region whereas neuroligin 1 is a type 1 membrane protein with a C-terminal transmembrane region. Together these findings suggest that neuroligin 1 is not the mammalian homolog of neurotactin.

Expression of Neuroligin 1 in Brain

RNA blotting experiments were performed to determine the site of expression of neuroligin 1. A single hybridizing mRNA of approximately 6 kb was observed only in brain (Figure 3A). No signal was detected in other tissues, even after prolonged exposure. In immunoblotting experiments with proteins from a variety of tissues, neuroligin 1 could also only be detected in brain (data not shown). In situ hybridization experiments using a specific oligonucleotide complementary to the neuroligin 1 mRNA showed a neuronal pattern of expression with weak hybridization to all brain areas (data not shown).

Enrichment of Neuroligin 1 in Synaptic Plasma Membranes

Subcellular fractionation of brain demonstrated that neuroligin 1 is enriched in synaptosomes similar to the postsynaptic density protein PSD95 (P2 in Figure 3B). Subfractionations of the synaptosomes into synaptic vesicles (LP2), cytosol (LS2), and synaptic plasma membranes (SPMs) show that neuroligin 1 also copurifies with PSD95 on SPMs. Both proteins are completely absent from synaptic vesicles. SPMs contain both pre- and postsynaptic elements and docked synaptic vesicles, resulting in the enrichment of most synaptic vesicle proteins in this fraction in addition to their presence in the synaptic vesicle fraction (except for rab3A, which dissociates probably during docking; Fischer von Mollard et al., 1991).

Splice Site-Specific Binding of Neuroligin 1 to β-Neurexins

Neuroligin 1 was purified in a single step from total brain by affinity chromatography on immobilized neurexin 1β containing a His_6-tagged C-terminus. To exclude potential artifacts caused by the method of immobilization of neurexin 1β or by the presence of a His_6 sequence and to examine the binding specificity of neuroligin 1 to different neurexins, we produced a series of recombinant neurexin–immunoglobulin G (IgG) fusion proteins and tested their ability to bind neuroligin 1 (Figure 4).

We produced 11 IgG fusion proteins to examine the binding specificity of neuroligin 1: one control IgG fusion protein consisting of the signal peptide of neurexin 1α fused to IgG (Figure 4, IGN control) and 10 neurexin–IgG fusion proteins in which the extracellular domains of α- or β-neurexins are fused to the Fc domain of human IgG after splice site 4 in the last G domain repeat (diagrammed in Figure 4A). Neurexins 1β and 2β were expressed with or without an insert in the alternatively spliced sequence 4 in the G domain (referred to as insert-negative and insert-positive β-neurexins; IGN 1β-1, 1β-3, 2β-3, and 2β-4 in Figure 4) and neurexin IIIβ without an insert at this site (IGN 3β-1 in Figure 4). In addition, four different insert-negative and insert-positive neurexin 1α–IgG fusion proteins were examined.

Neuroligin 1 binding to different neurexin–IgG fusion proteins was studied by testing the ability of the proteins to affinity purify neuroligin 1 from brain homogenates. Affinity chromatography on insert-negative β-neurexins resulted in the isolation of a family of closely spaced proteins of 110–116 kDa as the only proteins purified (see Coomassie blue–stained gel in top panel of Figure 4B). Binding of these proteins was Ca^{2+} dependent; no protein band was detected in the eluates from insert-positive β-neurexins. Immunoblotting confirmed that neuroligin 1 was a major

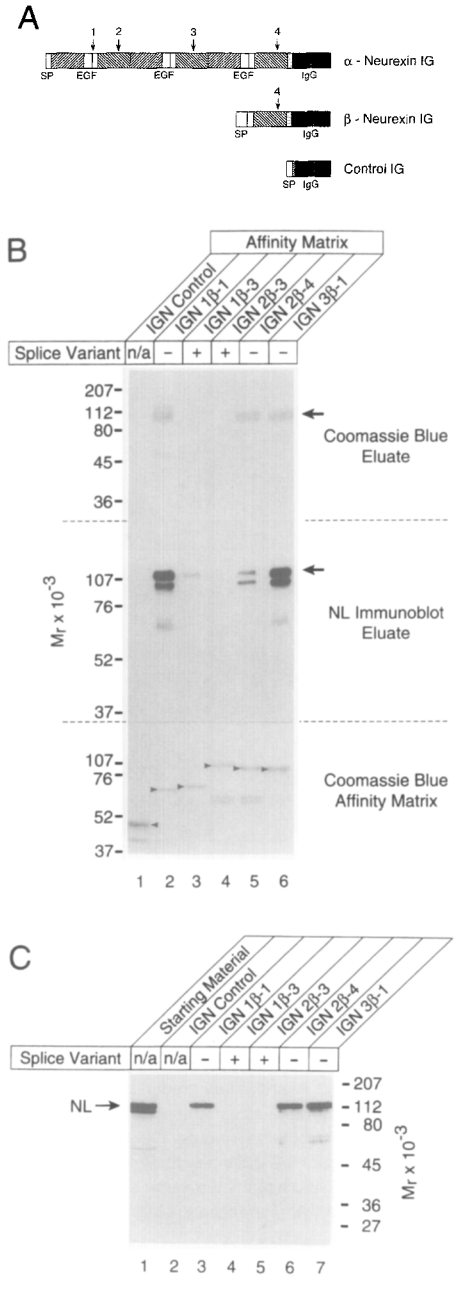

Figure 4. Neuroligin 1 Binding to β-Neurexin–IgG Fusion Proteins

(A) Structures of neurexin–IgG fusion proteins. IgG fusion proteins contain N-termini composed either of the extracellular domains of α-neurexins (α-neurexin IG) or of the extracellular domains of β-neurexins (β-neurexin IG) or of the neurexin 1α signal peptide only (control IG). The first four of the five canonical sites of alternative splicing in β-neurexins (Ullrich et al., 1995) are indicated by arrows and labeled 1–4. Only the N-terminal splice site of β-neurexins in the G domain (site 4) is present in the neurexin 1β constructs. The IgG domain is shown as a closed box, and different neurexin domains are shown as hatched boxes (SP, signal peptide; EGF, epidermal growth factor–like domain).

(B) Specificity of neuroligin 1 binding to β-neurexins with or without inserts in the G domain splice site. Control IgG fusion protein (lane 1) or IgG fusion proteins of neurexins 1β, 2β, and 3β without or with a splice site insert in the G domain repeat (lanes 2–6; for neurexin 3β, only the variant lacking an insert was analyzed) were used as affinity matrices for protein purification from total rat brain homogenate. The top panel depicts a Coomassie blue–stained gel of the eluate to demonstrate quantitative isolation of neuroligin 1 (arrows) by all three insert-negative β-neurexins but not by insert-positive β-neurexins. The middle panel shows an immunoblot of the eluates with an antibody to neuroligin 1. The bottom panel shows a Coomassie blue–stained gel of the IgG fusion proteins used (indicated by arrowheads).

(C) Affinity purification of neuroligin 1 expressed in COS cells by different β-neurexin–IgG fusion proteins. COS cells expressing transfected neuroligin 1 (lane 1) were solubilized in CHAPS and chromatographed on immobilized neurexin–IgG fusion proteins. Equivalent amounts of all fractions were analyzed by immunoblotting for neuroligin 1. Molecular weight standards are shown on the right.

component of the eluate from all insert-negative β-neurexins, although the eluates from the different β-neurexins are not stained equally by neuroligin antibodies (Figure 4B, middle). The purification of multiple protein bands that are not equally immunoreactive with neuroligin 1 antibodies indicates that there may be additional forms of neuroligin. Comparison of the Coomassie blue stain of the eluate with that of the affinity matrix (Figure 4B, top and bottom) suggests that neuroligin 1 binding to β-neurexins is stoichiometric. All of the binding observed is Ca^{2+} dependent (data not shown). In contrast with the binding of brain neuroligin 1 to insert-negative β-neurexins, no binding could be detected to insert-negative or insert-positive α-neurexins (data not shown). Together these results suggest that neuroligin 1 binds with high affinity only to β-neurexins that lack an insert in the alternatively spliced sequence in the G domain.

Recombinant Neuroligin 1 Reproduces the Binding Specificity of Native Neuroligin

To determine whether neuroligin 1 binds directly to β-neurexins or requires brain-specific proteins for binding, we used transfection to express neuroligin 1 in COS cells. A single 116 kDa immunoreactive band was detected in transfected COS cells, but not in control cells (data not shown). Membrane proteins from transfected COS cells were solubilized in CHAPS and chromatographed on immobilized neurexin–IgG fusion proteins as described above. As observed for brain homogenates, recombinant neuroligin 1 only bound to insert-negative β-neurexins but not to insert-positive β-neurexins (Figure 4C). Furthermore, no binding of recombinant neuroligin 1 to β-neurexins was detected (data not shown). Testing of multiple neuroligin 1 proteins that contain or lack inserts in the two alternatively spliced regions of neuroligin 1 showed that alternative splicing has no apparent effect on neurexin binding. These experiments confirm that we cloned a functionally active ligand for β-neurexins that only reacts with β-neurexins lacking an insert in the G domain splice site.

Presence of Native Neurexin-Neuroligin Complexes in Brain

Immunoprecipitations were performed to examine whether brain contains a complex of endogenous neurexins and neuroligin 1. Neurexins were immunoprecipitated from brain in the presence of Ca^{2+} using an antibody that reacts with the C-terminus of both α- and β-neurexins. This antibody was shown previously to immunoprecipitate α-latro-

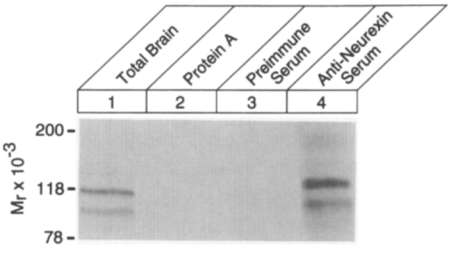

Figure 5. Coimmunoprecipitation of Neuroligin 1 with Neurexins from Rat Brain

Total rat brain homogenates in CHAPS (lane 1) were incubated in the presence of Ca^{2+} with protein A beads containing no antibodies (lane 2), with preimmune serum (lane 3), or with anti-neurexin antibodies (lane 3). Immunoprecipitates were eluted with EDTA and analyzed by immunoblotting for neuroligin 1. Only neurexin antibodies coimmunoprecipitate neuroligin 1, with the upper of the two bands observed corresponding to the size of neuroligin 1 expressed in COS cells; the lower band may be a proteolytic product or may correspond to a related protein. The slower migration on the gel of neuroligin 1 from the immunoprecipitate than from brain is probably due to the high levels of IgG in this sample. Numbers on the left indicate positions of molecular weight markers.

toxin-binding sites potently from rat brain (Hata et al., 1993a). Immunoprecipitates were washed with high salt and eluted with EGTA. As controls, protein A beads containing no antibodies or preimmune serum were processed identically. Immunoblotting of the eluates demonstrated that neuroligin 1 could be eluted from the immunoprecipitates obtained with the neurexin antibody but not from control immunoprecipitates (Figure 5). Two bands are observed similar to the purification of neuroligin 1 using neurexin–IgG fusion proteins, possibly because of proteolytic degradation or owing to the presence of multiple forms of neuroligin. Thus, native brain homogenates contain a complex between endogenous neurexins and neuroligin 1.

Primary Hippocampal Neurons Bind Recombinant β-Neurexins

To determine whether neuroligin 1 is a cell surface protein, we incubated transfected COS cells with β-neurexin–IgG fusion proteins. Only COS cells expressing neuroligin 1 bound insert-negative neurexin 1β at 4°C and 37°C and internalized it at 37°C during a 24 hr incubation. In contrast, the control IgG fusion protein did not bind (data not shown). This suggests that neuroligin 1 on the cell surface can bind β-neurexins, suggesting that developing neurons in culture may also expose binding sites. Therefore, we incubated primary hippocampal neurons from rat embryos with an insert-negative neurexin 1β–IgG fusion protein. At 4°C, specific binding of neurexin 1β in dotlike structures was observed, and longer incubations at 37°C resulted in their internalization (Figure 6; data not shown). Binding and internalization of β-neurexins were present all over the cell surface but concentrated on neurites.

Differential Expression of Neurexin Splice Forms in Brain

The specificity of neuroligin 1 binding to only one splice form of β-neurexins suggests a regulatory function for alternative splicing of neurexins in cell–cell interactions. This raises the question whether alternative splicing at the corresponding site of the neurexins is regulated. To address this, we investigated the distribution of alternative splicing of neurexin mRNAs in rat brain by in situ hybridization using specific oligonucleotides. Neurexins 1 and 2 had to be analyzed together in these experiments because their sequence similarity in this region did not allow synthesis of isoform-specific probes.

The hybridization patterns of splice site–specific oligonucleotides with rat brain sections reveal major differences in the regional distributions of spliced sequences (Figure 7). For example, the striatum, substantia nigra, and cerebellar nuclei exhibit much higher levels of insert-positive than of insert-negative neurexin 1 and 2 mRNAs whereas the CA3 region of the hippocampus and the cere-

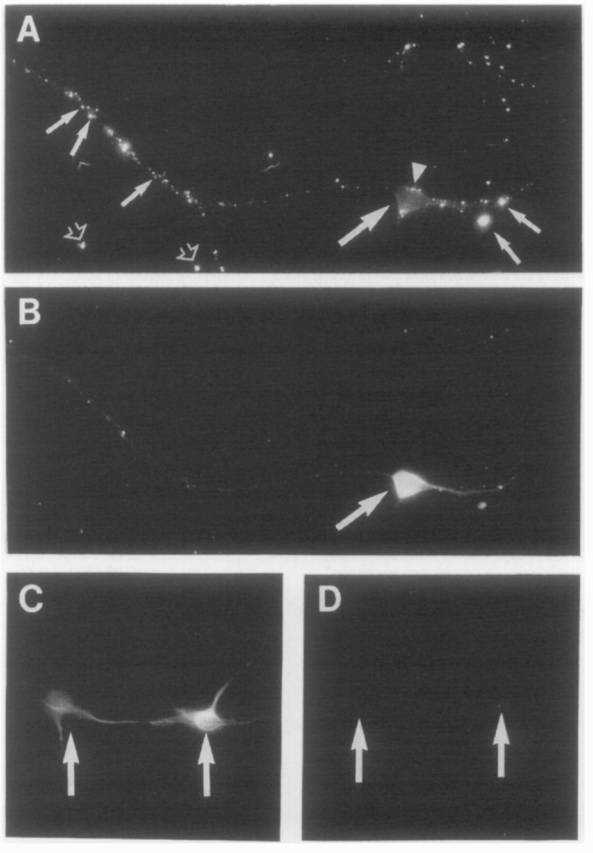

Figure 6. Binding of Neurexin 1β–IgG Fusion Protein to the Cell Surface of Cultured Hippocampal Neurons

Hippocampal neurons in primary culture were incubated with an insert-negative neurexin 1β fusion protein produced in transfected COS cells (A and B) or with corresponding material from control-transfected COS cells (C and D), fixed, and double labeled by immunofluorescence for human IgG to stain the β-neurexin fusion protein (A and C) and for MAP2 as a neuronal marker (B and D). Small arrows in (A) point to neurexin 1β dots of different sizes on neurites, large arrows to cell bodies, and open arrows to artifactual precipitates on the polylysine substrate used for the culture. Dots in the upper right quadrant in (A) belong to the neurites of a different neuron. Experiments using control IgG fusion proteins (Figure 4A) gave results similar to those shown in (C) and (D). (C) and (D) had exactly the same exposure times during photography as (A) and (B), respectively. Magnification, 118×.

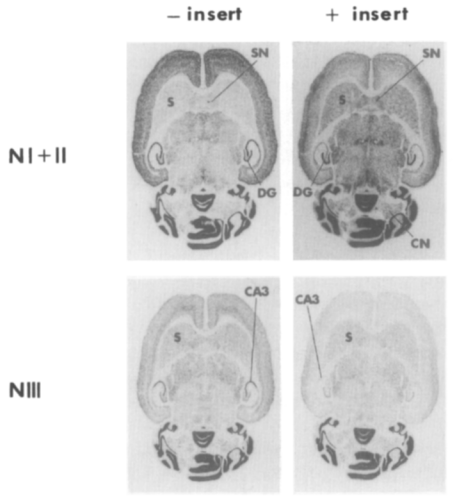

Figure 7. Differential Distribution in Rat Brain of Neurexin Splice Variants in the G Domain Repeat

Rat brain sections were hybridized with oligonucleotides specific for the insert-negative and insert-positive splice variants of neurexins 1 and 2 (NI + II) or 3 (NIII) at splice site 4, which regulates neuroligin 1 binding. Pictures show film autoradiograms exposed 3–6 weeks. Note the distinct differential expression of splice variants in areas such as the cerebellar nuclei (CN), the striatum (S), the substantia nigra (SN), the dentate gyrus (DG), and the CA3 region (CA3) of the hippocampal formation.

bral cortex show the opposite pattern. For neurexin 3, the most striking difference in distribution is observed in the hippocampus, where the pyramidal neurons of CA1 to CA4 have no detectable insert-positive mRNA but express high levels of insert-negative mRNA. Thus, alternative splicing at the site that regulates neuroligin 1 binding to β-neurexins is highly regulated.

Discussion

Neurexins are neuronal cell surface proteins characterized by a high degree of polymorphism that results from an unusually diverse pattern of alternative splicing. Using affinity chromatography on immobilized neurexin 1β, we have isolated a protein named neuroligin 1 that tightly binds to β-neurexins but not to α-neurexins. Neuroligin 1 interacts with all three β-neurexins, but it only binds to those β-neurexins that lack an insert in the alternatively spliced sequence in the G domain. Cloning of neuroligin 1 revealed that it constitutes a novel type 1 membrane protein with a large extracellular domain, a single transmembrane region, and a cytoplasmic tail. Data base searches showed that neuroligin 1 belongs to a class of proteins referred to as esterase domain proteins because of their homology to esterases such as acetylcholinesterase, although neuroligin 1 lacks the active site serine residue required for catalytic activity. Thus, a ligand for β-neurexins was discovered that only binds to one splice form of β-neurexins and is itself an intrinsic membrane protein on the neuronal cell surface.

Based on these data, we would like to propose that surface expression of neuroligin 1 and β-neurexins on neurons leads to tight interactions between these neurons. This recognition event is dependent on the alternative splicing of the β-neurexins in the G domain, which exhibits a distinct differential distribution in brain. α-Neurexins do not bind, although they also contain the site of alternative splicing that regulates β-neurexin binding, presumably because the N-terminal sequence unique to β-neurexins is involved in neuroligin 1 binding. Therefore, recognition among neurons mediated by the neurexin–neuroligin interaction is dependent on transcriptional regulation (expression of different β-neurexins that are heterogeneously distributed) and regulation of alternative splicing. This demonstrates an interaction between intrinsic membrane proteins that is regulated by alternative splicing. Interestingly, agrin, a secreted protein that contains G domains, is also alternatively spliced at two positions next to or in a G domain (Rupp et al., 1991; Ruegg et al., 1992). Alternative splicing of agrin regulates its biologic activity (Ruegg et al., 1992; Ferns et al., 1993), but no splice site–specific receptor for agrin has been identified.

The model for the function of β-neurexins and neuroligin 1 is supported by the following findings. First, neurexins and neuroligin 1 are both neuronal cell surface proteins. Second, neuroligin 1 only binds to one of two splice variants of β-neurexins in the G domain, a domain that also serves to mediate binding to cells in other proteins (Gehlsen et al., 1992). Third, the alternatively spliced sequence recognized by neurologin 1 is differentially distributed in brain. Fourth, a native complex between endogenous neurexins and neuroligin 1 is present in brain. α- and β-neurexins are expressed in many splice variants in brain that may give rise to more than 2000 proteins (Ullrich et al., 1995). Neuroligin 1 only binds to one splice variant of β-neurexins that is present in only half of the β-neurexins. Thus, neuroligin 1 is a ligand only for a small fraction of neurexins. Major additional ligands for neurexins remain to be discovered that could collaborate to determine the specifity of interactions between neurons.

The exact subcellular localizations of neurexins and neuroligin 1 and the place of their interaction are unknown. It is tempting to speculate that they may mediate interactions between neurons at the synapse. This hypothesis is suggested by the localization of neurexin 1 to synapses, the homology of neuroligin 1 to synaptic acetylcholinesterase, its enrichment in SPMs together with a postsynaptic density marker, and the Ca^{2+} dependence of neurexin–neuroligin binding since synaptic structures are dependent on extracellular Ca^{2+}. It would imply that neurexins are localized to the presynaptic terminal and neuroligin 1 to the postsynaptic side and that their interaction contributes to the organization of the synapse. The regulated expression of the alternatively spliced variant of β-neurexins that binds neuroligin 1 suggests that the β-neurexin–neuroligin 1 interaction may stabilize subclasses of synapses. The tight interaction of neuroligin 1 with β-neurexins could form a stable scaffold for other interactions, thereby increasing the specificity of interactions or specifying a defined sequence of interactions.

Experimental Procedures

Production of Recombinant Neurexins by Expression in Baculovirus and in COS Cells

Neurexin 1β was cloned into the baculovirus expression vector pVL1392 (Invitrogen) by fusing its extracellular domains at the SalI site (corresponding to residue 1339 in neurexin 1α; Ushkaryov et al., 1992) to a His_6 sequence followed by a stop codon. Recombinant baculoviruses were produced (Summers and Smith, 1987) and used to infect Sf9 cells cultured in serum-free medium (SF900-II, GIBCO). Recombinant neurexin 1β was purified from the culture medium 72 hr after infection by precipitation with 75% ammonium sulfate, by chromatography on concanavalin A–Sepharose, and by chromatography on Ni^{2+}/NTA–agarose (Qiagen). Neurexin 1β was eluted by 0.25 M imidazole, dialyzed, and purified further by anion exchange chromatography on MonoQ and by gel filtration on Superdex-200, after which the protein was electrophoretically pure (yield of 10 mg/l). Purified neurexin 1β was coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia) with 5 g of protein per liter of gel. Construction of α-neurexin–IgG and β-neurexin–IgG fusion protein constructs and production of protein by transfection in COS cells were performed as described by Ushkaryov et al. (1994).

Purification of Neuroligin 1 and Protein Sequencing

Rat brains (60 g) were homogenized in 0.25 l of buffer A (0.1 M NaCl, 4 mM KCl, 5 mM $CaCl_2$, 2.5 mM EDTA, 20 mM $NaHCO_3$, 20 mM Tris–HCl [pH 7.5], 0.1 g/l PMSF, 1 mg/l leupeptin, 1 mg/l pepstatin A, and 1 mg/l aprotinin). CHAPS was added to a final concentration of 2% (w/v) and dissolved under stirring at 4°C for 1 hr. Insoluble material was removed by low speed (2,000 × g) and high speed (80,000 × g) centrifugations. The supernatant (approximately 220 ml) was diluted with an equal volume of buffer A and chromatographed on the affinity columns (neurexin 1β–His_6 coupled to Sepharose, BSA coupled to Sepharose, and different IgG proteins attached to protein A–Sepharose) preequilibrated in buffer A containing 0.8% CHAPS. Columns were eluted with buffer B (1 M NaCl, 4 mM KCl, 5 mM $CaCl_2$, 2.5 mM EDTA, 20 mM $NaHCO_3$, 20 mM Tris–HCl [pH 7.5], 0.8% CHAPS) followed by buffer B containing 25 mM EDTA and 25 mM EGTA. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and by silver staining, Coomassie blue staining, and immunoblotting. For amino acid sequencing, neuroligin 1 was separated on an SDS gel, blotted, and digested with trypsin (Hata et al., 1993b). Sequences were obtained after high pressure liquid chromatography purification of tryptic fragments on an ABI amino acid sequencer.

cDNA Cloning, Construction of Expression Vectors, and COS Cell Expression of Neuroligin

The longest peptide sequence from neuroligin 1 (ELNNEILVPVIQFLGVPYAAPPT) was chosen to design short oligonucleotides complementary to its ends. Sequences, with letters in parentheses indicating redundant positions, are as follows: CGCGAGCTCAA(C,T)AA (C,T)GA(G,A)AT(A,T,C)(T,C)T and GGGCTGCAGCNGC(A,G)TANGGNACNCC. The cDNA sequence encoding the peptide was cloned by PCR using single-stranded rat brain cDNA as a template and sequenced. Multiple related sequences were obtained, indicating the possibility of multiple neuroligins. An oligonucleotide was designed (CGCCCATGGTCAATAACGAGATACTGGGCCCGGTCGTGCAGTTCTTGG) on the basis of the PCR sequence most closely related to the peptide sequence and used in a second set of PCRs with a fourth oligonucleotide based on the peptide sequence PYKELVDQ (sequence, TGCAGATCTTGGTCCAC[C,T]A[G,A][T,C]TC[C,T]TT[A,G]TANGG). A 0.8 kb product from this PCR encoding the correct sequence was used for screening rat cDNA libraries. Of the clones isolated, 19 were characterized by DNA sequencing, and six were fully sequenced. Expression vectors were created by subcloning two cDNA clones that either contain or lack the the alternatively spliced sequences (pNLig37a and pNLig62b) into pCMV-based expression vectors and transfected into COS cells (Ushkaryov et al., 1994).

Antibodies and Immunoprecipitations

Antibodies were raised against a synthetic peptide from the C-terminus of neuroligin 1 (sequence, CHPHPHPHSHSTTRV) coupled to keyhole limpet hemocyanin (Johnston et al., 1989). Aliquots (5 ml) of CHAPS-solubilized rat brain homogenates prepared as described above were incubated for 2 hr at 4°C with 20 μl of preimmune serum, anti-neurexin antibodies (Hata et al., 1993a), or no additions. After adding 50 μl of protein A–Sepharose (Pharmacia) preequilibrated with buffer A containing 1% CHAPS and 5 g/l BSA, samples were incubated for 14 hr. Beads were washed twice in buffer A containing 1% CHAPS and in buffer B and eluted in buffer B containing 25 mM EDTA and 25 mM EGTA. The eluate was precipitated with TCA and analyzed by SDS–PAGE and immunoblotting.

β-Neurexin–IgG Binding to Transfected COS Cells and Primary Hippocampal Neurons

Primary hippocampal neurons were cultured on polylysine-coated coverslips (Banker and Cowan, 1977). Coverslips after 4–10 days in vitro were transferred to 12-well dishes. IgGs and IgG fusion proteins purified from the media of COS cells transfected with control DNA or with neurexin 1β and control IgG fusion protein vectors were added to the culture medium. After 2 or 24 hr incubation at 4°C or 37°C, coverslips were rinsed with medium, fixed in 4% paraformaldehyde, 0.1 M phosphate buffer [pH 7.4], treated with 0.3% Triton X-100 (5 min), and blocked in 2% goat serum (1 hr). Cells were stained with a mouse monoclonal antibody against MAP2 (Sternberger) followed by fluorescein-conjugated goat anti-mouse antibodies and rhodamine-labeled goat anti-human IgG. Transfected COS cells were transferred to polylysine-coated coverslips 1 day after transfection, cultured for 2 days more, and then treated the same way as the hippocampal cultures.

In Situ Hybridizations

In situ hybridizations were performed (Ullrich et al., 1995) using rat brain cryostat sections and ^{35}S-labeled oligonucleotides of the following sequences: insert-negative neurexins 1 and 2, GAAGATTGTGAGCTGACGCCCTGCAGGGTATCGTTCGATGAC; insert-positive neurexins 1 and 2, ACTCGACCAAGTCGATATGGAATTCGCTGTCTAGCAATCGCCAG; insert-negative neurexin 3, GAAGATGGTT AGCTGCCGGCCTGTAGGATAATGCTCATTCAC; insert-positive neurexin 3, CAGGCCGATTATATTTGAAGGGGATTTTCTGTTTTACCATTTGG; and neuroligin 1, CTTCTGCAGGCATTCTACTAACTCTACTGTATCTGACACA. For controls, all hybridizations were performed with a 50- to 100-fold excess of unlabeled oligonucleotide to displace specific signals, resulting in a complete abolition of signal.

Miscellaneous Procedures

Subcellular fractionation of fresh rat brains was performed as described previously (Jones and Matus, 1974). SDS–PAGE, immunoblotting, and silver staining were performed using standard procedures and antibodies described previously (Johnston et al., 1989; Ushkaryov et al., 1992). RNA blots were purchased from Clontech and hybridized with uniformly labeled cDNA probes (Ushkaryov et al., 1992).

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