

Synaptic Arrangement of the Neuroligin/ β -Neurexin Complex Revealed by X-Ray and Neutron Scattering

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SUMMARY

Neuroligins are postsynaptic cell-adhesion proteins that associate with their presynaptic partners, the neurexins. Using small-angle X-ray scattering, we determined the shapes of the extracellular region of several neuroligin isoforms in solution. We conclude that the neuroligins dimerize via the characteristic four-helix bundle observed in cholinesterases, and that the connecting sequence between the globular lobes of the dimer and the cell membrane is elongated, projecting away from the dimer interface. X-ray scattering and neutron contrast variation data show that two neurexin monomers, separated by 107 Å, bind at symmetric locations on opposite sides of the long axis of the neuroligin dimer. Using these data, we developed structural models that delineate the spatial arrangements of different neuroligin domains and their partnering molecules. As mutations of neurexin and neuroligin genes appear to be linked to autism, these models provide a structural framework for understanding altered recognition by these proteins in neurodevelopmental disorders.

INTRODUCTION

Synaptic connectivity is tightly regulated during development in the central nervous system. Owing to the asymmetric nature of the synaptic connections, some of the molecules involved in partnering within the pre- and postsynaptic regions of the synapse have heterophilic recognition and adhesive properties (Ferreira and Paganoni, 2002). Neuroligins and neurexins show such properties; in fact, in vitro overexpression or RNAi knockdown of these respective post- and presynaptic proteins modulates clustering of associated synaptic proteins, suggest-

ing a critical role in forming and/or maintaining synapses (Scheiffele et al., 2000; Graf et al., 2004; Chih et al., 2005). In vivo, neuroligin function appears to be more critical for synapse maturation than for the initial formation of synaptic contacts (Varoqueaux et al., 2006). Polymorphisms of the coding regions of neurexin and neuroligin genes (including point mutations, truncations, and exon deletions) were recently found to be associated with autism spectrum disorders and mental retardation, indicating a strong genetic link to neurodevelopmental disorders (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005; Talebizadeh et al., 2006; Feng et al., 2006; Szatmari et al., 2007).

The neuroligins comprise a family of transmembrane proteins composed of an extracellular, N-linked glycosylated domain with strong sequence homology to acetylcholinesterase (AChE), a Ser-Thr-rich stalk domain that carries both N- and O-linked oligosaccharides (Ichtchenko et al., 1996; Bolliger et al., 2001; Hoffman et al., 2004), a single transmembrane domain, and a small, intracellular C-terminal domain. The neuroligins bind in vitro to both α - and β -neurexin (NX α and NX β) in a Ca²⁺-dependent manner (Ichtchenko et al., 1995; Boucard et al., 2005). A Ca²⁺-binding site has been identified in NX β , but the role of Ca²⁺ binding in the association and its physiological functions are unclear.

The selectivity of neuroligin/neurexin association is tightly regulated by a hierarchy of structural determinants. First, different neuroligin isoforms (NL1–4) are encoded by separate genes and have different affinities for NX β (Comoletti et al., 2006; Graf et al., 2006), suggesting that expression of individual neuroligin isoforms is associated with discrete synaptic pathways. Second, alternative splicing of mRNAs encoding both neurexins and neuroligins provides a basis for additional discrimination via the multiplicity of potential gene products (Graf et al., 2006; Chih et al., 2006; Comoletti et al., 2006). Finally, glycosylation and its processing of an alternatively spliced region in NL1 negatively regulate neurexin binding (Comoletti et al., 2003; Boucard et al., 2005), thus offering posttranslational control of selectivity of association.

Whereas the crystal structures of the second and sixth LNS (laminin/neurexin/SHBG-like) domains of NX α have been solved (Rudenko et al., 1999; Sheckler et al., 2006), only models based on the domain homology with AChE are currently available for the neuroligins. An initial monomeric homology model (Tsigelny et al., 2000) was refined by using experimental data on the cysteine connectivity and the sites of O- and N-linked oligosaccharide attachment of the extracellular domain of NL1 (Hoffman et al., 2004). This study confirmed that the neuroligins belong to the α/β -hydrolase fold family of proteins, a common fold shared by the cholinesterases and several other serine hydrolases. However, two additional N-linked glycosylation sites, two positions of alternative splicing, and the linkage of the disulfide bond between Cys512 and Cys546 in NL1 reveal complexity in the NL1 structure beyond the predictions based solely on AChE homology. Although the influence of splice inserts A and B on the recognition of neuroligin for NX β has been widely investigated (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006; Graf et al., 2006), their locations with respect to the interacting neuroligin/neurexin surface are not known. Using analytical ultracentrifugation data, the extracellular domains of the neuroligins were shown to exist as stable dimers (Comoletti et al., 2003, 2006), and a dimeric model based on the crystal structure of the dimeric AChE (Sussman et al., 1991; Bourne et al., 1995) was proposed (Dean et al., 2003). However, definitive data on the dimerization surface of the neuroligins and on the tertiary structure and orientation of the stalk domain with respect to the globular domain of the neuroligins and the postsynaptic membrane are lacking.

We report here the results of small-angle solution scattering experiments on the extracellular domains of the neuroligins and the complex formed between NL1 and NX β . X-ray scattering data provide overall shape information on the extracellular domains of the neuroligins and the initial structural definition of the O-linked glycosylated domain linking the extracellular domain to the transmembrane span. We also use the X-ray scattering data to refine our homology model for the NL1 dimer. A combination of X-ray scattering and neutron contrast variation data with *ab initio* and rigid-body modeling has yielded a structural model of the extracellular domain of NL1 complexed with NX β . Our models are presented in relation to the synaptic disposition of the complex, and, while of inherently low resolution, they provide an important structural framework for linking genetic information on mutated neurexins and neuroligins with neurodevelopmental disorders.

RESULTS

Protein Sample Quality for Small-Angle Scattering

Extraction of structural information on individual protein molecules from solution scattering data requires that all protein molecules in solution are identical (i.e., pure, monodisperse solutions) and sufficiently dilute such that there are no distance correlations between molecules. Distance correlations between molecules (usually due to

Coulombic repulsion) give rise to interparticle interference effects that suppress the small-angle scattering data and result in artificially small measured R_g values. These interparticle interference effects are linear with concentration and can be removed by extrapolation to infinite dilution. Hence, accurate structural analyses require data extrapolated to infinite dilution or measured at low enough concentrations to ensure that the interparticle interference effects are negligible.

The purity of our protein samples was established by SDS-PAGE and analytical gel filtration (Figures S1A and S1B; see the Supplemental Data available with this article online), and the monodispersity was verified by the scattering data. The observed decrease in R_g values for the NL1-638- Δ (A&B) and NL1/NX β complex (Table 1) as concentration is increased is evidence that the samples are monodisperse and that there is interparticle interference. Scattering profiles, $I(Q)$ versus Q , measured at the low-concentration limit, where interparticle interference effects are negligible, for each of the neuroligins are shown for comparison in Figure 1B. Additional evidence of sample monodispersity comes from the linearity of the Guinier plots within the Guinier region ($QR_g < 1.3$) (Figure 1B, inset), and the volumes determined for each neuroligin dimer by using the Porod invariant. These volumes show a close correspondence to the values estimated from the partial-specific volume and the NL1 dimer molecular weights determined by mass spectrometry (Comoletti et al., 2006).

Analysis of the Scattering Data of NL1 Is Consistent with the Expected Dimer Structure and Indicates that the Stalk Domain Is Extended

The $P(r)$ profile represents the probable distribution of interatomic distances within the molecule and provides some insight into the molecular structure independent of model structures. The $P(r)$ profile for the extracellular domain of NL1 truncated after the α/β -hydrolase fold domain at residue 638 (NL1-638) gives values for R_g and D_{max} of 42.7 ± 0.7 Å and ~ 130 Å, respectively (Figure 2A; Table 1). These values are consistent with previous hydrodynamic determinations of NL1 as a dimer (Comoletti et al., 2003, 2006). NL1-691 represents the entire extracellular domain of NL1, including a 53 amino acid segment carrying several O-linked oligosaccharides and an N-linked glycosylation site at position N662 (Hoffman et al., 2004). The presence of oligosaccharides, combined with an abundance of Pro residues, presumably maintains the peptide chain in a semirigid, bottlebrush-like structure, as demonstrated in the P-selectin molecule and the T cell coreceptor CD8 protein (Li et al., 1996; Merry et al., 2003). Inspection of the $P(r)$ profile of NL1-691, compared to NL-638 (Figure 2A), reveals that the main peak broadens and moves to larger r values: D_{max} increases by ~ 35 Å, and a low frequency of distances appears beyond ~ 130 Å, approximately where the $P(r)$ function of the shorter NL-638 mutant approaches zero. These changes indicate that the stalk domain extends away from the main globular domain (Figure 2A, inset). The prevalence of both N- and O-linked oligosaccharides in the C-terminal sequence is

Table 1. Scattering Data Statistics of the Neuroligins and the Neuroligin/Neurexin Complex

Sample	Protein Conc. ^a (mg/ml)	R_g (Å)	D_{max} (Å)	Porod Volume ^b ($10^3 \times \text{Å}^3$)	Calculated Volume ^c ($10^3 \times \text{Å}^3$)	MW ^d (kDa)
SSRL Data						
NL1-638-Δ(A&B)	1.8	42.5 ± 0.4	130	209 ± 20	198	130/151/144
NL1/NX complex	3.6	46.8 ± 0.2	155	*	275	181/201/199
University of Utah Instrument Data						
NL1-638-Δ(A&B)	13.2	38.3 ± 0.3				
	7.6	40.1 ± 0.5				
	4.1	41.4 ± 0.6				
	Inf. dilution	42.4 ± 0.6	130	208 ± 14	198	130/151/144
NL1-638	3.3	42.7 ± 0.7	130	250 ± 19	220	136/166/160
NL1-691	3.8	51.8 ± 1.0	165	255 ± 26	257	148/189/185
NL2-615	3.7	40.6 ± 0.6	130	178 ± 7	193	135/146/140
NL3-639	1.2	40.3 ± 0.7	130	164 ± 12	190	128/144/138
NL4-619	3.4	42.1 ± 0.6	135	199 ± 7	200	132/140/145
NL1/NX complex	19.7	40.9 ± 0.3				
	15.7	40.8 ± 0.2				
	9.8	44.0 ± 0.3				
	8.7	43.7 ± 0.4				
	6.6	44.5 ± 0.4				
	4.5	45.2 ± 0.5				
	3.8	47.7 ± 0.9	155	*		
	Inf. dilution	47.7 ± 0.8	155	*	275	181/201/199
Neutron Data, 42% D ₂ O						
NL1/deuterated NX complex	5.1	49.0 ± 2.0	155	n.a.	n.a.	n.a.

^a Concentrations for all of the NL constructs (except NL-691) are determined from I(0) analysis by comparison to a lysozyme standard measured in the same cell on the same day. The NL-691 concentration is from a Bradford assay. Concentrations for the complexes are all based on quantitative amino acid analysis. Infinite dilution refers to the results from the scattering data concentration series extrapolated to zero concentration.

^b Volumes calculated from the scattering data by using the Porod invariant (Porod, 1951). The asterisk indicates that the volumes for these data sets varied significantly with the Q max value chosen; thus, the uncertainty of these values is much larger than the propagated statistical errors quoted for the other volumes and are not considered to be reliable.

^c Calculated from the molecular weight (Da)/partial specific volume (v) where MW was determined from mass spectrometry measurements.

^d These molecular weight values were calculated as follows: from peptide sequence; from peptide sequence plus assuming full glycosylation; from mass spectrometry data on independent samples.

in accord with the larger volume of NL1-691 compared to NL1-638 (Table 1).

NL1-638-Δ(A&B) is the reference NL1 structure used for all of our modeling. This NL1 variant, which lacks both splice inserts A and B, which are present in NL-638, gives values for R_g and D_{max} of 42.5 ± 0.4 Å and ~130 Å, respectively (Figure 2B; Table 1, Stanford Synchrotron Radiation Laboratory [SSRL] data). The agreement between the low concentration measurement for NL-638-Δ(A&B) taken at SSRL and data from the same sample preparation taken

by using the University of Utah instrument and extrapolated to infinite dilution (Table 1) verifies that SSRL samples are a good approximation to infinite dilution. The $P(r)$ function determined for NL-638-Δ(A&B) extends to the same D_{max} value determined for NL1-638, indicating that the splice inserts do not affect the longest distances in the structure. There is a small increase in the frequency of vector lengths between ~50 and 90 Å for NL-638, consistent with the presence of the two additional loops folded onto each globular domain.

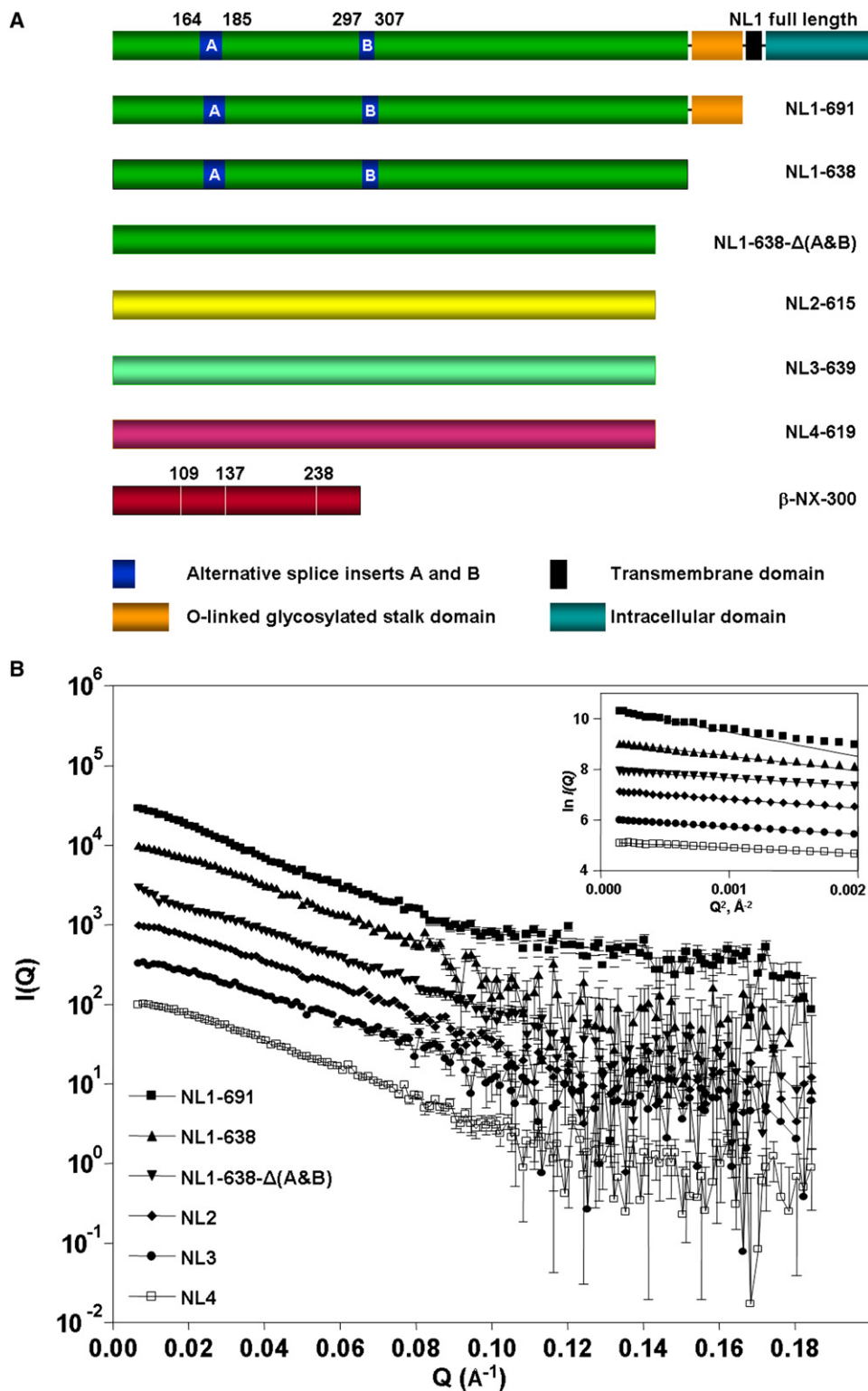


Figure 1. Schematic Representation of the Extracellular Domains of the Neuroligins and NX β and Scattering Profiles and Guinier Plots of the Neuroligins

(A) Domain organization of the full-length NL1 is shown on top. Different recombinant neuroligin isoforms were generated by truncation of the AChE-homologous domain, as shown by the numbers on the right side of each domain. The numbering on top of the full-length NL1 refers to residues flanking splice inserts A and B. The amino acid numbering of NL1–3 is according to [Ichtchenko et al. \(1996\)](#); that for NL4 is according to [Bolliger et al. \(2001\)](#). The numbers on the NX β construct refer to the mutations described in the text and are in accord with [Ushkaryov et al. \(1994\)](#).

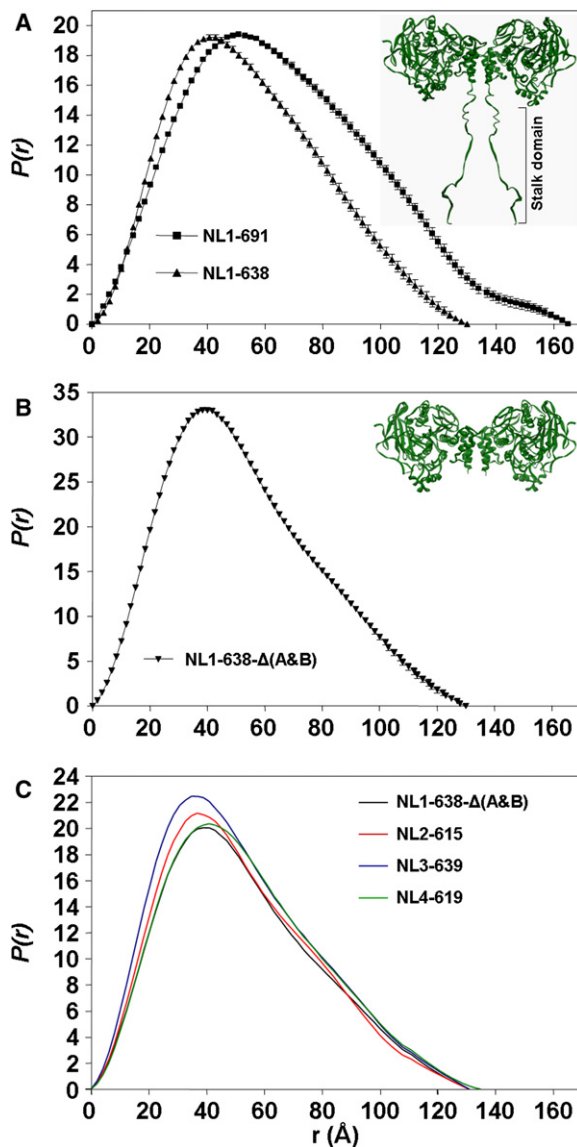


Figure 2. Pair Distance Distribution Functions of Various Neuroligin Constructs

(A) The $P(r)$ functions of two NL1 species, containing both splice inserts A and B and C-terminally truncated at positions 638 and 691. Areas under the $P(r)$ curves have been normalized by the square of the average molecular weight for the scattering particle. Inset: a model of NL1 truncated at position 691 with the stalk domain modeled in an extended conformation by using the cellulose Cel48F protein (PDB code: 1FBW) as the template is shown for comparison purposes only. Error bars show errors propagated from counting statistics.

(B) The $P(r)$ functions of NL1-638-Δ(A&B) calculated by GNOM from data shown in Figure 1B. Inset: dimeric NL1 model truncated at position 638. The statistical quality of the data can be assessed by the error bars; some estimated errors are smaller than the symbols. Error bars show errors propagated from counting statistics.

(C) The $P(r)$ functions of the four neuroligins, NL1–4. Symbols have been removed for clarity. Error bars are omitted for clarity.

Neuroligins 1–4 Form Dimers of Similar Dimensions with the Dimerization Domains Resembling Those of AChE

As expected from the high sequence identities (72%–77%) among the neuroligins (Ichtchenko et al., 1996; Bol-liger et al., 2001), our scattering data and associated parameters, such as the D_{max} and R_g values show that the extracellular domains of all of the neuroligins are similar in overall shape and dimensions, indicating that they share a common fold and have similar dimerization domains (Table 1). Inspection of the $P(r)$ functions of the soluble extracellular domains of NL1–4 (Figure 2C) reveals only minor deviations from our NL1 reference structure. Physical parameters and $P(r)$ profiles from scattering data collected on mouse AChE truncated at position 548 (Marchot et al., 1996) indicate that the NL structures are also very similar to the AChE structure, supporting our homology model as a reliable starting point for the solution structure of all of the neuroligins (Figure S2).

The starting model for rigid-body refinement against the NL1-638-Δ(A&B) data was obtained by manually docking two NL1 monomers, by using the AChE crystal structure as a guide. The crystal structure of *Torpedo californica* AChE shows two monomeric units connected by a four-helix bundle containing a cysteine bond, thus forming a covalent dimer (Sussman et al., 1991). The same dimerization motif was found in the crystal structures of mouse AChE lacking that C-terminal cysteine, indicating that dimerization of the AChEs does not require a covalent bond (Bourne et al., 1995). For our NL1-638-Δ(A&B) model, residues 450–462 and residues 621–634 made up the two helices that form the dimerization domain. This assembly was then optimized by simulated annealing/molecular dynamics structure refinement of the relative positions of the monomer units against X-ray scattering data (Figure 3). When compared to our initial homology model, the refined structure had one of the monomers rotated $\sim 20^\circ$ about the long axis of the dimer molecule, and, as a result, the atomic coordinates of the backbone atoms have ~ 6 Å root mean square distance displacement with respect to the initial coordinates (Figure 3).

Shape restoration, using ab initio methods, provides an alternate modeling approach to the interpretation of scattering data that has minimal assumptions about starting geometries. We therefore used ab initio shape restoration (implemented in the programs DAMMIN, DAMAVER, and DAMFILT) to derive the most probable shape that best fit the scattering data for NL1. Figure 3 shows the excellent agreement between this most probable shape and the atomic model derived from the rigid-body refinement. Similar results were obtained from shape-restoration calculations of AChE-548 (Figure S2) and for neuroligins 2–4 (Figure S3). The $P(r)$ function for NL1-691 exhibits significantly increased R_g and D_{max} values, which likely result from the C-terminal Ser-Thr-rich domain extension.

(B) Scattering profiles of the six neuroligin dimers examined in this study. Data come from samples with the lowest protein concentrations reported in Table 1. Profiles have been offset on the vertical axis for clarity. Inset: Guinier plots of the scattering profiles; solid lines represent the best fit through the Guinier regions of the neuroligins. The symbols are the same as those used in the main panel. Error bars represent counting statistics.

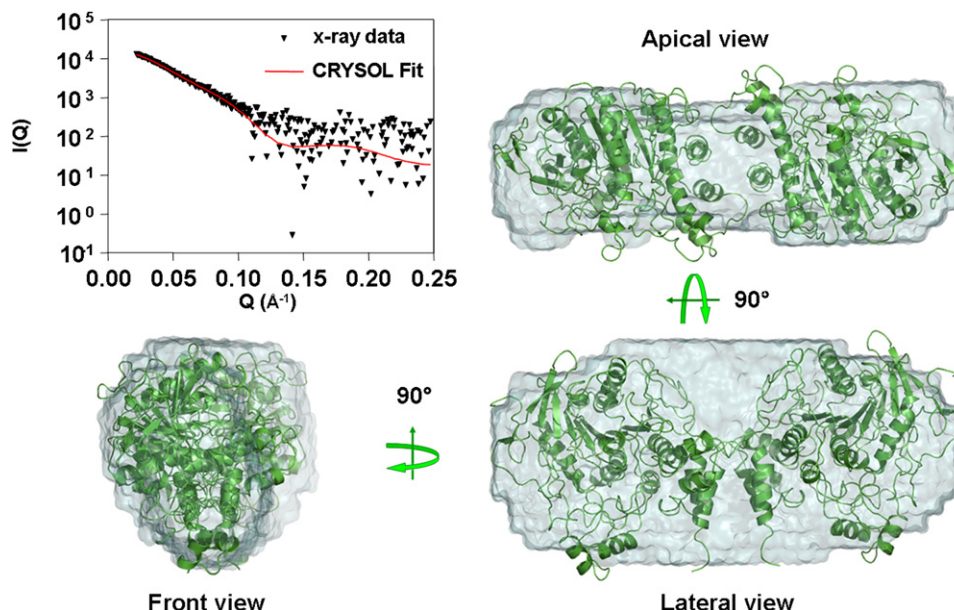


Figure 3. Ribbon Representation of the Dimeric NL1 Model Superimposed on the Ab Initio Shape-Restoration Envelope Derived from the Scattering Data

The DAMMIN shape-restoration envelope is shown as a transparent surface; green ribbon, NL1-638-Δ(A&B) structural model obtained after rigid-body refinement of the initial homology model against the scattering data, aligned by using SUPCOMB. Arrows indicate the direction and degree of each rotation used to obtain the different views. In the front view of the assembly, the two monomers are aligned so that only one unit is visible. The quality of the fit between the experimental data and the model profile calculated by using CRY SOL and the coordinates of the refined structure is shown in the inset ($\chi^2 = 1.117$). The apical view of the NL1 dimer shows the four-helix bundle that forms the dimerization interface of the dimer.

However, this feature was not resolved in the shape-restoration calculations, possibly due to its relatively narrow, single-chain structure and its likely segmental motion in the absence of cell membrane tethering.

Two NXβ Molecules Bind on Opposite Sides of the Long Axis of the NL1 Dimer in a Defined Orientation and with a Median Separation of 107 Å

As NL1-638-Δ(A&B) has the highest affinity for NXβ (Comoletti et al., 2006), it was chosen to study the complex. To ensure that measurements were performed on fully complexed samples (i.e., 2:2 stoichiometry), protein concentrations were determined by quantitative amino acid analysis and were checked by $I(0)$ analysis after data collection. To improve the statistical precision of the data used for modeling, we took the average of three independent X-ray scattering data sets measured at the low-protein concentration limit determined to exclude interparticle interference (Figures 4A and 4B). The corresponding $P(r)$ profile yielded R_g and D_{max} values of 47.7 ± 0.9 Å and ~ 155 Å, respectively (Table 1). The ~ 25 Å increase in D_{max} compared to the free NL1-638-Δ(A&B), accompanied by a small shift of the main peak (~ 40 – 45 Å) and an increase in intensity between ~ 60 Å and ~ 110 Å (Figure 4C, triangles), indicates that NXβ binds at the periphery of the NL1 dimer, distant from the dimer symmetry axis.

Ab initio shape restoration from the scattering data for the complex resulted in $\sim 50\%$ of the DAMMIN calculations converging to a single class of shapes that shows two clearly recognizable protrusions from the basic NL1

dimer shape in locations that are consistent with the binding data and with the NXβ separation distance obtained from the neutron contrast variation experiment (below). The remaining 50% of the conformations showed degenerate solutions that were inconsistent with these data. As this same degeneracy was observed when modeling theoretical intensity profiles calculated by using the program CRY SOL and the atomic model we derive for our complex (below), it appears that the degenerate solutions are artifacts of the reconstruction algorithm for this shape.

In order to determine unambiguously the positions of the NXβ subunits relative to the NL1 dimer, we required an experiment that could distinguish between the NL1 and NXβ components. The signal intensity in a small-angle scattering experiment is proportional to the square of the difference between the scattering densities of the particle and the solvent (the “contrast”). As isotopes of hydrogen ($^1\text{H} = \text{H}$) and deuterium ($^2\text{H} = \text{D}$) have different neutron “scattering powers,” the neutron scattering density of a protein, as well as its solvent, can be systematically varied by substituting H for D. Therefore, deuterated NXβ was prepared, and stoichiometric amounts of deuterated NXβ were combined with NL1-638-Δ(A&B) in the presence of 2 mM Ca^{2+} . By acquiring neutron scattering data from the complex in a 42% D_2O solution, the protonated NL1 subunits were effectively “solvent matched”; that is, they had the same approximate mean neutron scattering density as the solvent and thus did not contribute significantly to the scattering signal, allowing for direct measurement of the scattering from the NXβ units. The $P(r)$ curve extracted

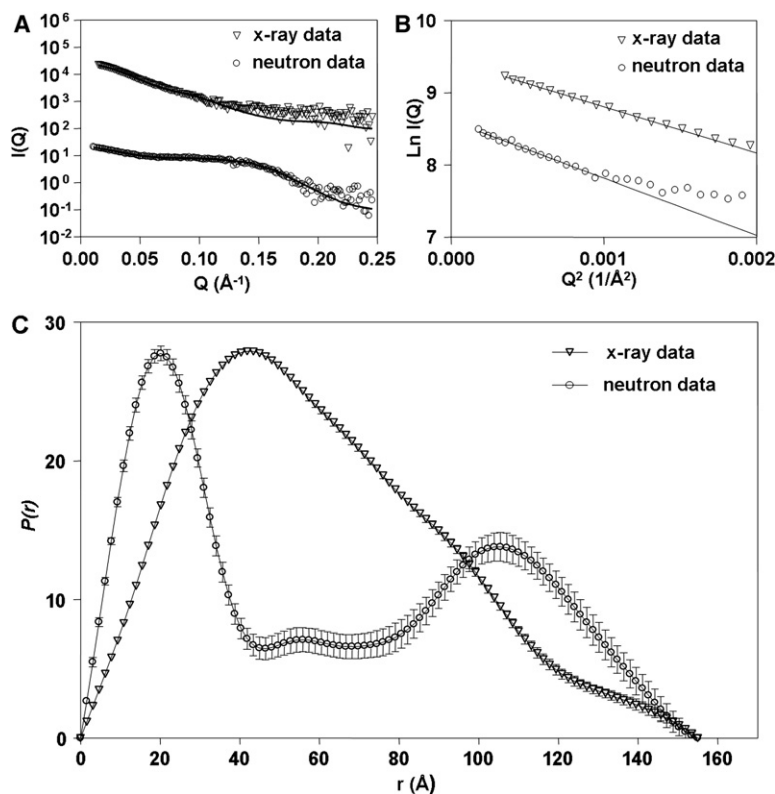


Figure 4. Scattering Intensities, Guinier Plots, and Pair Distance Distribution Functions of the NL1/NX β Complex

(A and B) Scattering intensity and Guinier plots, respectively, of the X-ray and neutron data and their fits to the profiles calculated by using CRY SOL and CRYSON and the coordinates of the final complex. The χ^2 values for the fit to the X-ray and neutron scattering data are 1.454 and 0.584, respectively.

(C) Comparison of the $P(r)$ functions of the NL1/NX β complex derived from X-ray and neutron scattering data for the sample in 42% D₂O buffer. As the nondeuterated component (NL1-638- Δ (A&B)) has a very weak contribution to the neutron scattering signal at this D₂O concentration, the $P(r)$ function is dominated by the bound, deuterated NX β components (open circles). Error bars show errors propagated from counting statistics.

from the neutron scattering data (Figure 4C, open circles) exhibits two maxima at ~ 20 Å and 107 Å: the first corresponds to the most frequent interatomic distance within the individual NX β units, and the second corresponds to the most frequent interatomic distance between the two NX β subunits. The combined X-ray and neutron scattering data thus provide the approximate binding locations for the NX β subunits and the separation of their centers of mass: a powerful set of restraints for refining a solution structure of the complex.

The initial starting point for the rigid-body modeling procedure was obtained by placing the NX β subunits proximal to the NL1-638- Δ (A&B) dimer to best fit the low-resolution density reconstruction of the complex and according to known biochemical constraints (Comoletti et al., 2003, 2006; Boucard et al., 2005; Graf et al., 2006). A series of rotations of the NX β subunits within this model allowed us to sample a diverse set of initial geometries for the rigid domain refinement. Refinement against X-ray data from all starting geometries converged to only two alternate models related by the 180° rotation of the NX β monomers, both of which had the rim of the β sheet sandwich opposite the N and C termini of NX β as the site of contact with NL1. The scattering data thus proved to be sensitive to the orientation of the discoid shape of the NX β subunits, allowing us to select the best fitting of the two for additional refinement against the X-ray scattering data while restraining the NX β /NX β inter-domain separation according to the neutron scattering data. This final calculation produced a structure in which

the NX β monomers are positioned in agreement with the available mutagenesis data (Comoletti et al., 2006; Graf et al., 2006) and the C termini of the NL1 and NX β subunits facing opposite directions, consistent with the complex bridging pre- and postsynaptic surfaces (Figure 5).

DISCUSSION

Using small-angle solution scattering, we determined the three-dimensional structural model of the AChE-like domain of the neuroligins, showing that all four neuroligins have similar overall shapes and dimensions, as evidenced by their $P(r)$ functions, R_g and D_{max} values, and three-dimensional shape reconstructions. The stalk region connecting the globular domain of the neuroligins with their transmembrane domain is elongated and projects away from the globular domain. This fundamental information on the neuroligin structure, based on X-ray scattering and neutron contrast variation experiments, enables us to present the first, to our knowledge, three-dimensional structural model of NL1 in a complex with NX β and to place it in the context of the synapse.

The very good fit of X-ray scattering data with the neuroligin model, the similarity with the solution structure determined for the AChE whose crystal structure has been extensively studied, and the correspondence among their physical parameters strongly indicate that the neuroligin dimer is held together by the four-helix bundle typical of the α/β -hydrolase fold (Bourne et al., 1995). This arrangement of the dimerization domain results in each stalk

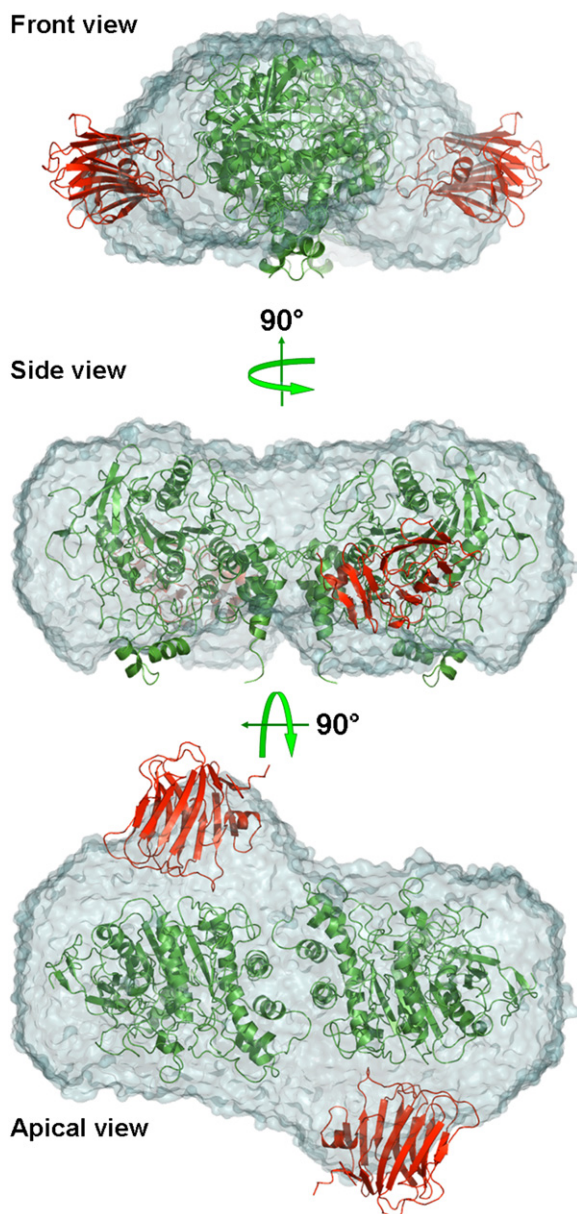


Figure 5. Ribbon Representation of the Refined Structures for the NL1/NXβ Complex Superimposed on the Ab Initio Shape-Restoration Envelopes of the Scattering Data

Surface representations for the shapes derived with DAMMIN are shown as a semitransparent surface; green ribbon, NL1-638-Δ(A&B) model; red ribbon, NXβ structure. Alignment of the envelopes and model structure is done with SUPCOMB. Arrows indicate the direction and degree of each rotation.

region emerging from the same side of the dimer. The very small differences in distance distributions ($P(r)$) between the spliced and unspliced forms of NL1 indicates that splice inserts A and B in the NL1 fold are within the main globular domain, consistent with their role in neurexin association (Boucard et al., 2005; Comoletti et al., 2006).

A puzzling element of the structure of these heterophilic adhesion proteins is the relatively small mass of their ex-

tracellular domains when compared with other transmembrane synaptic proteins such as N-cadherins, L1, and NCAM (Piechotta et al., 2006). As such, the globular extracellular domains of the neurexins and neuroligins, even when associated as a complex, are too small to span the synaptic space. However, the O-linked glycosylated sequence with its relatively rigid, bottle-brush-like structure offers an extended stalk region intervening between the AChE-homologous domain and membrane-spanning regions. This structure may only be observable in solution studies, as filamentous and glycosylated domains generally preclude crystallization. As the neurexins also have a similar stalk region between their LNS and transmembrane domains, it is expected that both stalk regions act as intervening lines of variable extension linking the sequence emerging from the membrane to the globular domains. Hence, the *trans*-synaptic space can be spanned from presynaptic and postsynaptic membrane tethers.

In LNS domains, the metal-binding pocket is located at the rim of the β sheet sandwich opposite the N and C termini (Rudenko et al., 2001). In NXβ, alanine mutations of D137 and N238, residing on this rim, lack synaptogenic activity (Graf et al., 2006), possibly participating in the binding of Ca^{2+} . On the NL1 surface, mapping NXβ-binding determinants revealed that the region flanking splice insert B (including E297 and K306) (Boucard et al., 2005; Comoletti et al., 2006) is the focal point for NXβ binding, consistent with our solution scattering data on the NL1/NXβ complex. In addition, the neuroligin surface near splice insert B is well removed from the other three sites of glycosylation, that were shown to have no influence on neurexin association (Comoletti et al., 2003). Based on secondary-structure predictions, it was suggested that splice insert 4 of neurexin forms two helices located in the long loop connecting strands $\beta 10$ and $\beta 11$ (Rudenko et al., 1999). Although initial reports based on in vitro pull-down and cell-associated studies described splice insert 4 as the main switch for NL1 binding (Ichtchenko et al., 1995, 1996), NXβ interaction with the neuroligins is modulated in a more complex manner (Boucard et al., 2005; Graf et al., 2006; Comoletti et al., 2006). Consistent with our results, the insertion point of splice insert 4 in the LNS domain does not directly contact the NL1/NXβ interface.

In the synapse, orientation of the neuroligins and neurexins in the complex requires that their C-terminal regions extend the O-linked glycosylated domains in opposite directions. Since both N and C termini emerging from the LNS domain were disordered in the crystal structure (Rudenko et al., 1999), establishing an orientation a priori of the C-terminal end of the NXβ monomer was not possible. Nevertheless, our rigid-body structure refinement of X-ray and neutron solution scattering data favors one particular orientation of the NXβ monomers in which interfacial residues of NL1 and NXβ are defined by the available mutagenesis data. In a recently published model (Dean and Dresbach, 2006), it is proposed that two neurexin monomers bind to the apical ends of the two neuroligin monomers, an arrangement inconsistent with the available

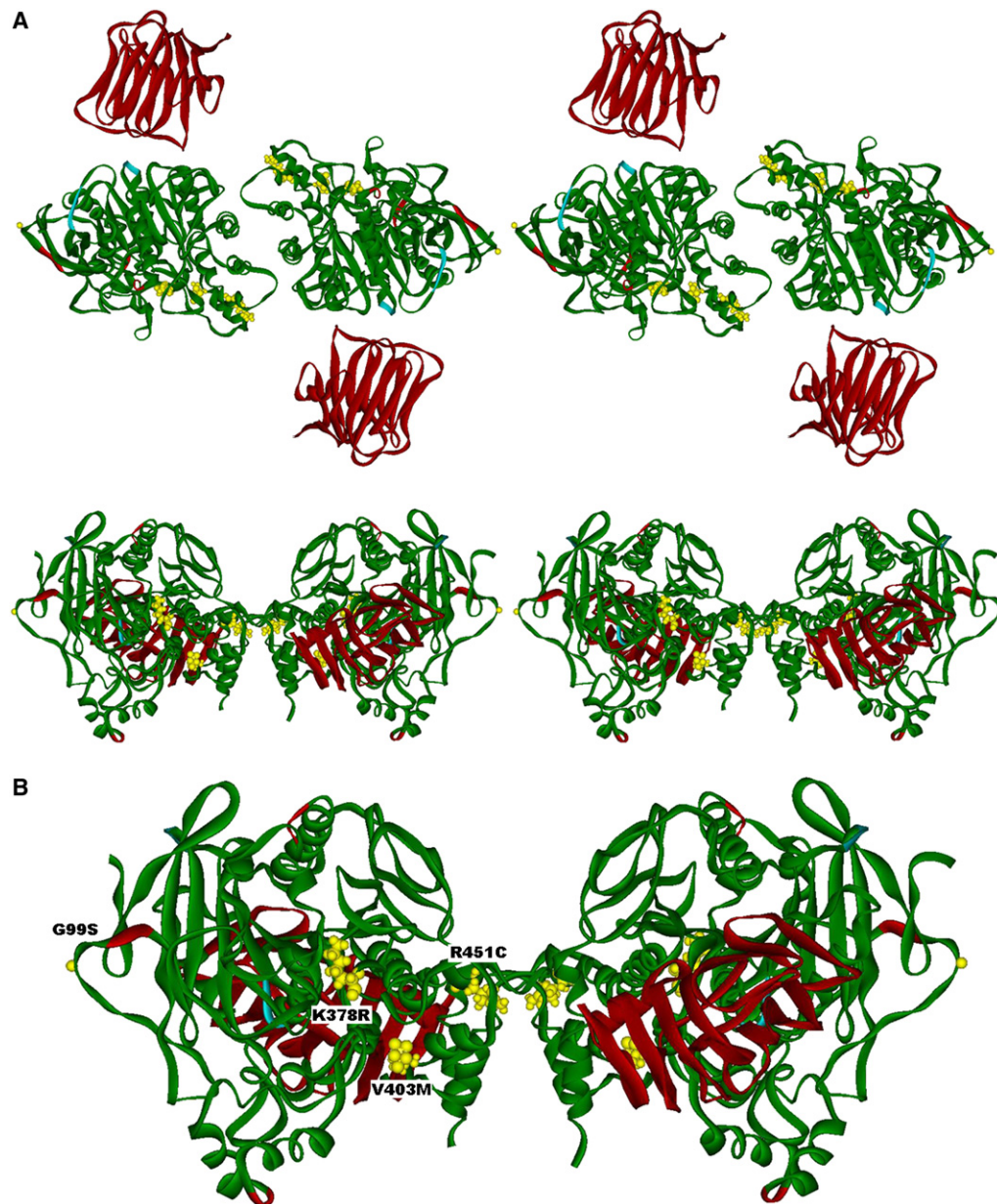


Figure 6. Stereomage of the NL1/NX β Complex

(A and B) (A) Top panel, view of the complex from the presynaptic side; lower panel, side view of the complex. Green, neuroigin dimer; red, neurexin. Neuroligin-3-R451C (Jamain et al., 2003) and Neuroligin-4-G99S, -K378R, and -V403M (Yan et al., 2005) are shown as yellow amino acid side chains and are identified in (B). Two flanking residues on each side of splice inserts A and B are shown in cyan; N-linked glycosylation sequences are shown in red.

biochemical data, as well as the X-ray and neutron scattering data reported in this study. Synaptic-differentiation studies suggest that a minimum of four neurexin molecules have to associate to obtain presynaptic activation in vitro (Dean et al., 2003), whereas solution studies clearly demonstrate that a stable 2:2 complex is formed (Comolletti et al., 2006). These results imply that formation of larger clusters of these synaptic proteins may occur through intracellular domains.

An R451C mutation of the *NL3* gene and three mutations of the *NL4* gene (G99S, K378R, and V403M) were

recently found in autistic patients (Jamain et al., 2003; Yan et al., 2005). These amino acids are highly conserved in the neuroigin family and are located in the AChE-like domain. Upon mapping these mutations in our structural model of the NL1/NX β complex, it is interesting to note that NL4-K378R, NL4-V403M, and NL3-R451C cluster in a region opposite the NX β -binding region on each monomer and relatively close to the symmetry axis of the dimer. In contrast, NL4-G99S is found on a surface loop at the extreme end of the long axis of the neuroigin dimer (Figure 6). Owing to their positions with respect to the NX β -binding

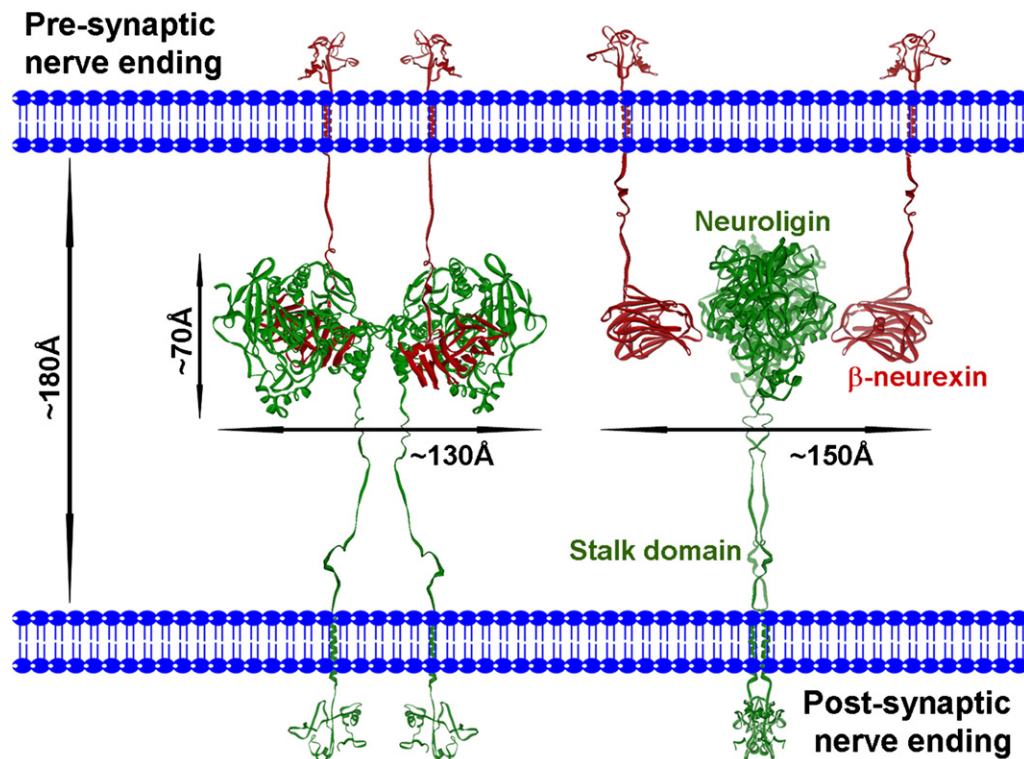


Figure 7. Model of the NL1/NX β Assembly in the Synapse

The neuroligin/neurexin complex is tethered to the pre- and postsynaptic membranes through their respective O-linked glycosylated regions that have been drawn extended to position the complex in the synaptic space. Arrows specify the approximate sizes of the synaptic cleft and the complex. Intracellular domains of both neuroligin and neurexin have no conformational assignment, since they appear unstructured in homologous proteins (Zeev-Ben-Mordehai et al., 2003).

interface, we predict that these mutations do not directly influence neuroligin/neurexin association. Although the R451C shows reduced NX β binding (Comoletti et al., 2004), it was recently shown that the mutation restricts processing through the endoplasmic reticulum probably because of local protein misfolding and an inability to oligomerize (De Jaco et al., 2006).

Conclusions

Glycosylation of the neuroligins and the extended stalk region has rendered a crystallographic solution of the structure of the extracellular portion of the neuroligins and their complex with NX β a challenging endeavor. Thus, we turned to solution scattering to determine the molecular shapes of the individual molecules in order to enhance our understanding of the complex formation within the synaptic space environment. Accordingly, solution scattering obviates the constraints dictated by the crystal packing (Heidorn and Trewhella, 1988). The presented structural models for NL1 and NL1/NX β are of inherently low resolution and are based on initial AChE homology as a reasonable starting point for the neuroligin dimer, for which there is good supporting evidence. Our study thus has afforded additional analysis of unstructured neuroligins, and the model of the neurexin and neuroligin associated in the synaptic space (Figure 7) provides

a structural framework for analyzing the disposition of these molecules within the synaptic space.

EXPERIMENTAL PROCEDURES

Expression Vectors and Mutagenesis

FLAG-NL1–NL4 cDNAs, devoid of both transmembrane and intracellular domains, were used to make the proteins NL1-638, NL2-615, NL3-639, and NL4-619, as described previously (Comoletti et al., 2006). NL1-638 contained both splice inserts A and B, whereas NL2-615, NL3-639, and NL4-619 did not contain either splice insert. NL1-638 was also engineered devoid of splice inserts A and B (NL1-638- Δ (A&B)). Soluble NL1-691 containing both splice inserts A and B and the O-linked glycosylated stalk domain was prepared by introducing a stop codon at Tyr692 (Comoletti et al., 2003). All mutations were obtained by using the QuikChange Mutagenesis Kit (Stratagene, San Diego, CA) and were verified by both restriction digests and DNA sequencing. The GST-cleavable β -neurexin construct was described elsewhere (Comoletti et al., 2004, 2006). Soluble mouse AChE (AChE-548) was constructed by introducing a stop codon at Cys549 of mouse AChE-GPI as described previously (Marchot, et al., 1996).

Neuroligin and Neurexin Expression and Purification

To purify each soluble neuroligin, tissue culture medium containing the secreted protein was passed over an M2 anti-FLAG affinity column (Sigma) (Comoletti et al., 2003). Samples were concentrated by microfiltration with Microcon 30YM (Millipore) and were separated by analytical gel filtration with a Superdex 200 HR 10/30 column (GE Healthcare). SDS-PAGE gels stained with Simply Blue Safestain

(Invitrogen, Carlsbad, CA) were used to check for purity and the absence of degradation products. For neutron scattering experiments, deuterated NX β was expressed in BL21 *E. coli* (Invitrogen, Carlsbad, CA) by using Spectra 9 deuterated media (Spectragases, Branchburg, NJ) and was purified as described (Comoletti et al., 2004, 2006). To establish the level of neurexin deuteration, mass spectrometry on purified recombinant NX β was performed as described (Comoletti et al., 2006). The deuterated NX β had a mass of 26,085 Da, indicating that all nonexchangeable hydrogens were deuterated (Figure S1B).

Quantitative Amino Acid Analysis

Protein concentrations were estimated by amino acid composition on a Beckman 6300 analyzer after hydrolysis in 5.7 N HCl containing 0.1% phenol in vacuum, at 110°C. Only reliably quantified amino acids (Ala, Arg, Asx [Asn + Asp], Glx [Gln + Glu], Ile, Leu, Lys, Phe, and Val) were used to estimate the protein concentration of samples analyzed by small-angle scattering.

Initial Homology Models of Neuroligins

Structural models of the extracellular domain of the neuroligins were generated with the programs Homology and InsightII (Accelrys, Inc.) by using the crystal structure of mouse AChE (PDB code: 1MAH) as a template. The structure of each neuroligin was then energy minimized for 10,000 iterations by using the distance-dependent dielectric constant with the program Discover (Accelrys, Inc.).

X-Ray Scattering Data Acquisition

X-ray scattering data for NL1-638- Δ (A&B) and its complex with NX β were acquired at the SSRL, beamline 4-2 (Tsuruta et al., 1998) at 20°C. The 20 keV radiation was incident in a pinhole configuration and a sample-to-detector distance of 1.5 m; all data processing was done by using the standard beamline 4-2 data-reduction procedures. X-ray scattering data were also collected by using the X-ray instrument now at the University of Utah described in Heidorn and Trehwella (1988). Samples measured on this instrument were maintained at 11°C.

The scattering from an exact solvent blank (containing 10 mM HEPES [pH 7.4], 2 mM CaCl₂, 150 mM NaCl; taken from the ultrafiltrates from concentration steps) was subtracted from each sample scattering profile to yield the protein molecules' scattering. Intensity data were reduced to $I(Q)$ versus Q by using standard procedures to correct for detector sensitivity and background signal. For NL1-638- Δ (A&B) and its complex with NX β , concentration series were measured and data were extrapolated (linearly) to infinite dilution to eliminate interparticle interference and establish at what low-concentration limit interparticle interference effects are negligible. All modeling analyses were done on samples that were at these low concentration limits. Correct stoichiometry for the complex was assured by using protein concentration values derived from quantitative amino acid analysis for NL1-638- Δ (A&B) and NX β to form a 2:2 complex.

Neutron Scattering Data Acquisition

Small-angle neutron scattering data were acquired for the NL1/NX β complex with protonated neuroligin and deuterated neurexin subunits in 42% D₂O solvent by using the NG3 instrument at the Center for Neutron Research at the National Institute of Standards and Technology (Gaithersburg, MD). To minimize possible protein aggregation, samples of the complex (5.2 mg/ml protein) were spun at 20,000 $\times$ g for 30 min prior to loading the cell. The incident neutron average wavelength, λ , was 5.82 ± 0.04 Å, with 15% $\Delta\lambda/\lambda$. Data were collected by using two geometries: 2.0 m sample-to-detector distance (2 hr data collection time), and a 5.0 m sample-to-detector distance (4 hr data collection time). The first setting gives an accessible Q range of 0.020 – 0.330 Å⁻¹, while the second gives a range of 0.008 – 0.095 Å⁻¹. Data were processed by using standard data-reduction software of the NG3 instrument. The interparticle interference term in the measured neutron scattering data was estimated to be less than 0.05% and therefore has negligible effect on the scattering param-

eters. This estimate is based on using the Percus-Yevick closure for the structure factor calculation as implemented in GIFT (Fritz et al., 2000).

X-Ray and Neutron Scattering Data Analysis

For an initial assessment of data quality, Guinier plots were evaluated for the expected linearity by using the program PRIMUS (Konarev et al., 2003). The pair distance distribution function, $P(r)$, was calculated by using the indirect Fourier transform method of Svergun (1992) as implemented in the program GNOM; the $P(r)$ function was required to go to zero at $r = 0$ and $r = D_{max}$, the maximum linear dimension of the scattering particle. GNOM was also used to correct for slit geometry of the X-ray source at University of Utah. Physical parameters determined for all neuroligins and the NL1/NX β complex are shown in Table 1. All statistical fittings shown were judged as excellent by the fit criteria of GNOM. The fits of the final model structures to the X-ray and neutron scattering data were evaluated with CRY SOL (Svergun et al., 1995) and CRYSON software (Svergun et al., 1998), respectively, with corrections for the wavelength and geometry smearing as appropriate.

Structure Modeling from the Scattering Data

Low-resolution ab initio shapes for each neuroligin isoform and for the NL1/NX β complex that best fit the scattering profiles from GNOM were generated as assemblies of dummy atoms by using the program DAMMIN (Svergun, 1999) enforcing C₂ symmetry. As described in Results, these calculations for the NL1/NX β complex gave degenerate solutions. Removal of the symmetry constraint did not help resolve this degeneracy, and it further increased the number of model parameters that could have the effect of overfitting the data. Ten independent models were aligned and averaged by using the program DAMAVER (Volkov and Svergun, 2003). The program DAMFILT (Volkov and Svergun, 2003) was used to filter the models; the mean volume of the constituent runs was set as the cutoff to produce the average model (Figures 3 and 5). Finally, the coordinates of the homology-based dimeric model were superimposed on the filtered DAMMIN model by using the program SUPCOMB (Kozin and Svergun, 2001).

Refined atomic models of both the neuroligin dimer and the entire NL1/NX β complex were obtained by rigid-body molecular dynamics refinement against small-angle scattering data by using the CNS program (Brunger et al., 1998) supplemented by a small-angle X-ray scattering fitting module (Grishaev et al., 2005). First, SSRL X-ray scattering data in the Q range between 0.022 Å⁻¹ and 0.247 Å⁻¹ were used for the NL1 dimer structure refinement. The AChE-based homology model of the dimer was used as a starting point in the refinement; the geometries of the individual domains were fixed throughout the protocol, and the C₂ symmetry of the dimer was enforced. Subsequently, X-ray data from the University of Utah instrument, in the Q range from 0.020 Å⁻¹ to 0.300 Å⁻¹, were used for further refinement; D_{max} was set to 150 Å for desmearing the data to remove effects of the instrument slit geometry. In all calculations for the complex, the structure of the NL1 dimer was kept identical to the refined dimer geometry obtained in the previous stage. NX β was fixed at the previously determined X-ray structure coordinates (PDB code: 1C4R), and the C₂ symmetry of the entire complex was enforced. In order to remove any potential bias from a particular orientation of the NX β units within the complex, four starting geometries were generated with the NX β monomers rotated in 90° steps around the vector perpendicular to the NL1 surface at the site of NX β contact. These initial geometries had the NX β residues 109, 137, and 238 facing the NL1 units, in agreement with published results (Graf et al., 2006). At the final stage of refinement, the separation between the centers of mass of the neurexin subunits was restrained to 107 Å, from the SANS data acquired in 42% D₂O. Final three-dimensional models were made by using PyMOL software version 0.97 (DeLano, 2004). All cited errors are based on propagated counting statistics only.

NL1-638- Δ (A&B) used in the complex characterization has three N-linked glycosylation sites. As we have previously studied the occupancy and type of carbohydrate in recombinant NL1 (Hoffman et al.,

2004), we considered ways to account for sugars. However, due to the facts that glycosylation in neuroligins is not clustered in a single region and that the sugars are intrinsically flexible and highly hydrated, we were unable to improve the quality of our fits to the scattering data by attempting to account for them. Of note, we do not see evidence for them in the shape-restoration models, suggesting that their contributions to the scattering are not significant. We thus conclude that their contributions cannot be measured above the background signal.

Supplemental Data

Supplemental Data include figures showing the characterization of the purified proteins employed in the study, the X-ray scattering data and analysis of soluble AChE, and the molecular envelopes of neuroligins 2–4 determined by using the scattering data and the ab initio shape restoration and are available at <http://www.structure.org/cgi/content/full/15/6/693/DC1/>.

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REFERENCES

- 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 a 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 neuroligins to α - and β -neurexins. *Neuron* 48, 229–236.
- Bourne, Y., Taylor, P., and Marchot, P. (1995). Acetylcholinesterase inhibition by fasciculin: crystal structure of the complex. *Cell* 83, 503–512.
- Brunger, A., Adams, P., Clore, G., DeLano, W., Gros, P., Grosse-Kunstleve, R., Jiang, J., Kuszewski, J., Nilges, M., Pannu, N., et al. (1998). Crystallography and NMR system: a new software for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 54, 905–921.
- Chih, B., Engelman, H., and Scheiffele, P. (2005). Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307, 1324–1328.
- Chih, B., Gollan, L., and Scheiffele, P. (2006). Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. *Neuron* 51, 171–178.
- Comoletti, D., Flynn, R.E., Jennings, L.L., Chubykin, A., Matsumura, T., Hasegawa, H., Südhof, T.C., and Taylor, P. (2003). Characterization of the interaction of a recombinant soluble neuroligin1 with neurexin-1 β . *J. Biol. Chem.* 278, 50497–50505.
- Comoletti, D., De Jaco, A., Jennings, L.L., Flynn, R.E., Gaietta, G., Tsigelny, I., Ellisman, M.H., and Taylor, P. (2004). The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. *J. Neurosci.* 24, 4889–4893.
- Comoletti, D., Flynn, R.E., Boucard, A.A., Demeler, B., Schirf, V., Shi, J., Jennings, L.L., Newlin, H.R., Südhof, T.C., and Taylor, P. (2006). Gene selection, alternative splicing, and post-translational processing regulate neuroligin selectivity for β -neurexins. *Biochemistry* 45, 12816–12827.
- Dean, C., and Dresbach, T. (2006). Neuroligins and neurexins: linking cell adhesion, synapse formation and cognitive function. *Trends Neurosci.* 29, 21–29.
- 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.
- De Jaco, A., Comoletti, D., Kovarik, Z., Gaietta, G., Radic, Z., Lockridge, O., Ellisman, M.H., and Taylor, P. (2006). A mutation linked with autism reveals a common mechanism of endoplasmic reticulum retention for the α , β -hydrolase fold protein family. *J. Biol. Chem.* 281, 9667–9676.
- DeLano, W. (2004). The PyMOL Molecular Graphics System (<http://www.pymol.org>).
- Feng, J., Schroer, R., Yan, J., Song, W., Yang, C., Bockholt, A., Cook, E.H., Jr., Skinner, C., Schwartz, C.E., and Sommer, S.S. (2006). High frequency of neurexin 1 β signal peptide structural variants in patients with autism. *Neurosci. Lett.* 409, 10–13.
- Ferreira, A., and Paganoni, S. (2002). The formation of synapses in the central nervous system. *Mol. Neurobiol.* 26, 69–79.
- Fritz, G., Bergmann, A., and Glatter, O. (2000). Evaluation of small-angle scattering data of charged particles using the generalized indirect Fourier transformation technique. *J. Chem. Phys.* 113, 9733–9740.
- 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 β LNS domain. *J. Neurosci.* 26, 4256–4265.
- Grishaev, A., Wu, J., Trehwella, J., and Bax, A. (2005). Refinement of multi-domain protein structures by combination of solution small angle X-ray scattering and NMR data. *J. Am. Chem. Soc.* 127, 16621–16628.
- Heidorn, D.B., and Trehwella, J. (1988). Comparison of the crystal and solution structures of calmodulin and troponin C. *Biochemistry* 27, 909–915.
- Hoffman, R.C., Jennings, L.L., Tsigelny, I., Comoletti, D., Flynn, R., 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.
- Ichtenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Südhof, T.C. (1995). Neuroligin 1: a splice site-specific ligand for β -neurexins. *Cell* 81, 435–443.
- Ichtenko, K., Nguyen, T., and Südhof, T.C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J. Biol. Chem.* 271, 2676–2682.
- Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I.C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C.,

- et al. (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat. Genet.* 34, 27–29.
- Konarev, P.V., Volkov, V.V., Sokolova, A.V., Koch, M.H.J., and Svergun, D.I. (2003). PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Cryst.* 36, 1277–1282.
- Kozin, M.B., and Svergun, D.I. (2001). Automated matching of high- and low-resolution structural models. *J. Appl. Crystallogr.* 34, 33–41.
- Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M.P., Raynaud, M., Ronce, N., Lecomnier, E., Calvas, P., et al. (2004). X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am. J. Hum. Genet.* 74, 552–557.
- Li, F., Erickson, H.P., James, J.A., Moore, K.L., Cummings, R.D., and McEver, R.P. (1996). Visualization of P-selectin glycoprotein ligand-1 as a highly extended molecule and mapping of protein epitopes for monoclonal antibodies. *J. Biol. Chem.* 271, 6342–6348.
- Marchot, P., Ravelli, R.B., Raves, M.L., Bourne, Y., Vellom, D.C., Kanter, J., Camp, S., Sussman, J.L., and Taylor, P. (1996). Soluble monomeric acetylcholinesterase from mouse: expression, purification, and crystallization in complex with fasciculin. *Protein Sci.* 5, 672–679.
- Merry, A.H., Gilbert, R.J., Shore, D.A., Royle, L., Miroshnychenko, O., Vuong, M., Wormald, M.R., Harvey, D.J., Dwek, R.A., Classon, B.J., et al. (2003). O-glycan sialylation and the structure of the stalk-like region of the T cell co-receptor CD8. *J. Biol. Chem.* 278, 27119–27128.
- Piechotta, K., Dudanova, I., and Missler, M. (2006). The resilient synapse: insights from genetic interference of synaptic cell adhesion molecules. *Cell Tissue Res.* 326, 617–642.
- Porod, G. (1951). Die Röntgenkleinwinkelstreuung von dichtgepackten kolloiden Systemen. *Kolloid-Z* 124, 83–114.
- Rudenko, G., Nguyen, T., Chelliah, Y., Sudhof, T.C., and Deisenhofer, J. (1999). The structure of the ligand-binding domain of neurexin-1 β : regulation of LNS domain function by alternative splicing. *Cell* 99, 93–101.
- Rudenko, G., Hohenester, E., and Muller, Y.A. (2001). LG/LNS domains: multiple functions - one business end? *Trends Biochem. Sci.* 26, 363–368.
- Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 10, 657–669.
- Sheckler, L.R., Henry, L., Sugita, S., Sudhof, T.C., and Rudenko, G. (2006). Crystal structure of the second LNS/LG domain from neurexin 1 α : Ca²⁺-binding and the effects of alternative splicing. *J. Biol. Chem.* 281, 22896–22905.
- Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991). Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* 253, 872–879.
- Svergun, D.I. (1992). Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Cryst.* 25, 495–503.
- Svergun, D.I. (1999). Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys. J.* 76, 2879–2886.
- Svergun, D.I., Barberato, C., and Koch, M.H.J. (1995). CRY SOL – a program to evaluate X-ray solution scattering of biological macromolecules from atomic coordinates. *J. Appl. Cryst.* 28, 768–773.
- Svergun, D.I., Richard, S., Koch, M.H., Sayers, Z., Kuprin, S., and Zaccai, G. (1998). Protein hydration in solution: experimental observation by X-ray and neutron scattering. *Proc. Natl. Acad. Sci. USA* 95, 2267–2272.
- Szatmari, P., Paterson, A.D., Zwaigenbaum, L., Roberts, W., Brian, J., Liu, X.Q., Vincent, J.B., Skaug, J.L., Thompson, A.P., Senman, L., et al. (2007). Mapping autism risk loci using genetic linkage and chromosomal rearrangements. *Nat. Genet.* 39, 319–328.
- Talebizadeh, Z., Lam, D.Y., Theodoro, M.F., Bittel, D.C., Lushington, G.H., and Butler, M.G. (2006). Novel splice isoforms for NLGN3 and NLGN4 with possible implications in autism. *J. Med. Genet.* 43, 21–28.
- Tsigelny, I., Shindyalov, I.N., Bourne, P.E., Sudhof, T.C., and Taylor, P. (2000). Common EF-hand motifs in cholinesterases and neuroligins suggest a role for Ca²⁺ binding in cell surface associations. *Protein Sci.* 9, 180–185.
- Tsuruta, H., Reddy, V.S., Wikoff, W.R., and Johnson, J.E. (1998). Imaging RNA and dynamic protein segments with low-resolution virus crystallography: experimental design, data processing and implications of electron density maps. *J. Mol. Biol.* 284, 1439–1452.
- Ushkaryov, Y.A., Hata, Y., Ichtchenko, K., Moomaw, C., Afendis, S., Slaughter, C.A., and Sudhof, T.C. (1994). Conserved domain structure of β -neurexins. Unusual cleaved signal sequences in receptor-like neuronal cell-surface proteins. *J. Biol. Chem.* 269, 11987–11992.
- Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Sudhof, T.C., and Brose, N. (2006). Neuroligins determine synapse maturation and function. *Neuron* 51, 741–754.
- Volkov, V.V., and Svergun, D.I. (2003). Uniqueness of ab initio shape determination in small-angle scattering. *J. Appl. Cryst.* 36, 860–864.
- Yan, J., Oliveira, G., Coutinho, A., Yang, C., Feng, J., Katz, C., Sram, J., Bockholt, A., Jones, I.R., Craddock, N., et al. (2005). Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients. *Mol. Psychiatry* 10, 329–332.
- Zeev-Ben-Mordehai, T., Rydberg, E.H., Solomon, A., Toker, L., Auld, V.J., Silman, I., Botti, S., and Sussman, J.L. (2003). The intracellular domain of the *Drosophila* cholinesterase-like neural adhesion protein, gliotactin, is natively unfolded. *Proteins* 53, 758–767.