Structural insights into the exquisite selectivity of neurexin/neurologin synaptic interactions

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The extracellular domains of neuroligins and neurexins interact through Ca^{2+} to form flexible *trans*-synaptic associations characterized by selectivity for neuroligin or neurexin subtypes. This heterophilic interaction, essential for synaptic maturation and differentiation, is regulated by gene selection, alternative mRNA splicing and post-translational modifications. A new, 2.6 Å-resolution crystal structure of a soluble neurexin-1β-neuroligin-4 (Nrx1β-NL4) complex permits a detailed description of the Ca^{2+} -coordinated interface and unveils concerted positional rearrangements of several residues of NL4, not observed in neuroligin-1, associated with Nrx1β binding. Surface plasmon resonance analysis of the binding of structure-guided Nrx1β mutants towards NL4 and neuroligin-1 shows that flexibility of the Nrx1β-binding site in NL4 is reflected in a greater dissociation constant of the complex and higher sensitivity to ionic strength and pH variations. Analysis of neuroligin mutants points to critical functions for two respective residues in neuroligin-1 and neuroligin-2 in governing the affinity of the complexes. Although neuroligin-1 and neuroligin-2 have pre-determined conformations that respectively promote and prevent Nrx1β association, unique conformational reshaping of the NL4 surface is required to permit Nrx1β association.

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Introduction

The post-synaptic neuroligins and presynaptic neurexins are transmembrane adhesion proteins whose extracellular domains interact to form *trans*-synaptic associations (Ichtchenko *et al*, 1996; Nguyen and Südhof, 1997; Gerrow and El-Husseini, 2006; Dalva *et al*, 2007). Neuroligins and neurexins are required for the maturation of glutamatergic excitatory and GABAergic inhibitory synapses, rather than for establishing the initial contact between neurons. Indeed, their over-expression in non-neuronal cells is sufficient to induce differentiation of co-cultured neurons (Scheiffele *et al*, 2000; Fu *et al*, 2003; Graf *et al*, 2004; Nam and Chen, 2005), whereas knockout mice for the neuroligin or neurexin genes display no major defect in synapse formation (Missler *et al*, 2003; Varoqueaux *et al*, 2006). The essential function of the neuroligins is further highlighted by the identification of mutations related to autism spectrum disorders and mental retardation in the neuroligin-3 (NL3) and neuroligin-4 (NL4) isoforms (Jamain *et al*, 2003; Laumonnier *et al*, 2004; Yan *et al*, 2005; Talebizadeh *et al*, 2006; Zhang *et al*, 2009). Proper central nervous system function depends in part on the ratio between excitatory and inhibitory synapses, and mutations in neuroligin structure may alter this parameter (Cline, 2005; Levinson and El-Husseini, 2005; Tabuchi *et al*, 2007; Südhof, 2008).

Five neuroligin genes have been identified in humans (NL1–5) and four in rodents (Ichtchenko *et al*, 1996; Philibert *et al*, 2000; Bolliger *et al*, 2001, 2008). The crystal structures of the extracellular cell adhesion domains of rodent NL1 and NL2 and human NL4 were solved recently (Araç *et al*, 2007; Fabrichny *et al*, 2007; Chen *et al*, 2008; Koehnke *et al*, 2008). The overall architecture of this domain falls within the α/β -hydrolase fold exemplified in the acetylcholinesterase (AChE) structure. When compared with the AChE subunit, distinct features such as a more hydrophobic dimerization interface, different conformations of surface loops surrounding the vestigial active centre and distinct electrostatic surface potentials are also evident. Furthermore, alternative RNA splicing for all neuroligins except NL4 yields isoforms containing or lacking inserts at splice sites A and B (SSA and SSB) (Ichtchenko *et al*, 1996; Bolliger *et al*, 2001).

The different isoforms of neuroligins exhibit different localization patterns in brain. Whereas NL1 and NL2 are predominantly present in glutamatergic and GABAergic synapses, respectively, NL3 is present in both types of synapses (Song *et al*, 1999; Varoqueaux *et al*, 2004;

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Budreck and Scheiffele, 2007). The low expression levels of NL4 (<5% of the total neuroligins in adult mouse brain) precluded ascertaining its precise synaptic localization (Varoqueaux *et al*, 2006). The majority of point mutations in neuroligins that relate to autism spectrum disorders reside within the AChE-like domain of NL3 and NL4. The molecular basis underlying their involvement in congenital brain disorders remains to be investigated, but the remote locations of these mutations from the neurexin-binding site preclude direct alteration at the neurexin-binding surface. Rather, these mutations likely affect neuroligin function through defective folding and trafficking (Comoletti *et al*, 2004; Zhang *et al*, 2009) or interactions with yet unidentified partner(s).

In humans, three neurexin genes are under the control of two promoters, which lead to long neurexins- α (Nrx α) and short neurexins- β (Nrx β) (Ushkaryov *et al*, 1992). Alternatively spliced inserts in the extracellular domain of neurexins could generate over 2000 possible isoforms (Rowen *et al*, 2002; Tabuchi and Südhof, 2002). The Nrx α extracellular sequences contain six laminin-neurexin-sex (LNS) hormone-binding globulin domains separated by three epidermal growth factor (EGF) domains, whereas Nrx β s are composed of a short specific N-terminal sequence followed by a single LNS domain, corresponding to the sixth LNS domain of Nrx α . This LNS domain common to two forms of neurexins is responsible for the interaction with neuroligins (Boucard *et al*, 2005) and may contain an insert at the alternative splicing site 4 (SS4). The crystal structures of the LNS domains of Nrx1 β and Nrx2 β and of the LNS-2 and LNS-4 domains of Nrx1 α reveal a similar lectin-like-fold made of a β jelly roll with a Ca²⁺-binding site located on the hypervariable-loop edge, near the SS4 position (Rudenko *et al*, 1999; Sheckler *et al*, 2006; Koehnke *et al*, 2008; Shen *et al*, 2008). Emerging evidence indicates that variations in copy number and rare variants within the *NRXN1* and *NRXN3* genes contribute to autism spectrum disorder susceptibility and mental retardation (Feng *et al*, 2006; The Autism Genome Project Consortium, 2007; Kim *et al*, 2008; Yan *et al*, 2008; Glessner *et al*, 2009).

As neuroligin and neurexin interactions have a crucial function in synapse validation and regulation (Chubykin *et al*, 2007), molecular-based studies are essential to understanding the determinants that govern complex formation and function. The large number of different isoforms of both partners makes a systematic *in vitro* analysis and thorough *in vivo* transgene studies laborious. Nevertheless, *in vitro* studies revealed that an intricate recognition code controls the Ca²⁺-dependent interaction between different neuroligin and neurexin isoforms. In broad outline, the NL1 interaction with Nrx β promotes formation of glutamatergic excitatory synapses, whereas NL2 in contact with Nrx α induces assembly of GABAergic inhibitory synapses (Boucard *et al*, 2005; Chih *et al*, 2006; Graf *et al*, 2006; Kang *et al*, 2008). Either interaction is further regulated by alternative splicing of both partners, ionic strength or N-linked glycosylation of neuroligins (Comoletti *et al*, 2003, 2006; Chen *et al*, 2008).

After the initial study of small angle X-ray and neutron scattering by Comoletti *et al* (2007), the molecular bases for recognition of neuroligins by neurexins have been approached at atomic resolution with crystal structures of Nrx1 β –NL1 and Nrx1 β –NL4 complexes (Araç *et al*, 2007; Fabrichny *et al*, 2007; Chen *et al*, 2008). These studies have

revealed the position and orientation of the bound Nrx1 β at the neuroligin surface and confirmed the requirement for Ca²⁺ at the complex interface. They also provide initial templates to understand the dynamic interaction network between different neuroligin and neurexin isoforms. For example, the spatial locations of SSB in NL1 and SS4 in Nrx1 β , both proximal to the binding interface, may explain partial inhibition of complex formation in splice variants.

Despite the availability of structural templates, the determinants of selective recognition between neuroligins and neurexins remain partially unsolved. For example, we earlier proposed that the very low affinity of Nrx1 β for NL2 ($K_D \sim 8.8 \mu\text{M}$) (Comoletti *et al*, 2006) might be related to the presence of a bulky Gln side chain at position 475 in the neurexin-binding site of NL2, in place of Gly500 in NL1 and Gly464 in NL4 (Fabrichny *et al*, 2007), but experimental data to confirm this are lacking. As well, the Nrx1 β affinity for NL1 is about four-fold higher than for NL4 ($K_D = 29$ and 132 nM , respectively) (Comoletti *et al*, 2006), but the structural basis for this difference remains unclear. Finally, the limited resolution of our initial structure of the Nrx1 β –NL4 complex (Fabrichny *et al*, 2007) precluded a detailed comparison with structures of the related Nrx1 β –NL1 complex or of free NL1 (Araç *et al*, 2007; Chen *et al*, 2008).

We have solved a new, 2.6 Å-resolution structure of the Nrx1 β –NL4 complex that permits precise positioning of all side chains at the complex interface, including those involved in Ca²⁺ coordination. Comparison with the structure of unbound NL4, solved at a similar resolution (Fabrichny *et al*, 2007), unveils conformational and positional rearrangements of loops and side chains at the NL4 surface upon Nrx1 β binding. Moreover, we used site-directed mutagenesis and surface plasmon resonance (SPR) to (i) explore in real time the dependency of Nrx1 β binding to NL1, NL4 and NL2 as a function of ionic strength and pH; (ii) analyse the relative contributions of structure-guided Nrx1 β mutants to complex formation and stability and (iii) determine the energetic contributions of two different interfacial residues selected from the structures, His294 in NL1 and Gln475 in NL2, in governing Nrx1 β binding. Our comprehensive analysis shows that whereas NL1 and NL2 are respectively preset conformationally to bind and not bind Nrx1 β , conformational reshaping at the NL4 surface is required for accommodating Nrx1 β .

Results and discussion

Improved accuracy of the Nrx1 β –NL4 complex structure

The overall 2.6 Å-resolution structure of the Nrx1 β –NL4 complex is similar to earlier structures of the same complex (3.9 Å; Fabrichny *et al*, 2007) and of Nrx1 β –NL1 complexes (2.4 and 3.5 Å; Araç *et al*, 2007; Chen *et al*, 2008) (Figure 1A). The extracellular domain of NL4 is composed of a twisted central β sheet surrounded by multiple α -helices, typical of the α/β -hydrolase fold. Two NL4 subunits related by a two-fold symmetry axis assemble as a non-covalent anti-parallel dimer, through a tightly packed four-helix bundle made of helices $\alpha_{7,8}^2$ and the C-terminal helix α_{10} from each subunit. The Nrx1 β -binding site on NL4, which consists of three surface loops encompassing residues His267–Gly271, Gln359–Asp366 and Gln462–Pro466, is located on the face opposite to the Cys-loop (Figure 1A), an equivalent to the long Ω loop at the active site gorge entrance of AChE

(cf. Fabrichny *et al*, 2007). The extracellular domain of Nr1β is composed of two anti-parallel β sheets of six and seven β strands, arranged in a jelly roll β-sandwich typical of lectin and lectin-like domains (Rudenko *et al*, 1999). Nr1β is bound with its β-sandwich oriented perpendicular to the long axis of the NL4 dimer and its concave side facing the NL4 four-helix bundle. The NL4-binding site on Nr1β corresponds to the hypervariable-loop edge, opposite to the N- and C-termini (Figure 1A). Compared with the structures of unbound and NL1-bound Nr1β (Araç *et al*, 2007; Chen *et al*, 2008; Shen *et al*, 2008), no conformational change is detected in the NL4-bound Nr1β molecule.

This new structure of the Nr1β–NL4 complex permits a detailed description of the interface, notably in the vicinity of the Ca^{2+} coordination sphere. Ca^{2+} lies central to the interface where it is coordinated by six ligands and adopts a typical octahedral geometry (Figure 1C). Four coordinating

oxygen atoms are provided by Nr1β residues (Asn238, Asp137, Val154, Ile236) and two others by water molecules. NL4 interactions with Ca^{2+} only involve residues Glu361 and Gln359 through water-mediated contacts. The position and coordination of Ca^{2+} in the Nr1β–NL4 complex are similar to those in unbound Nr1β and the Nr1β–NL1 complex (Araç *et al*, 2007; Chen *et al*, 2008; Shen *et al*, 2008), arguing for conservation of the Ca^{2+} -binding site geometry in the neurexin–neuroligin interaction.

The binding interface is flat and covers a surface area of $\sim 620 \text{ \AA}^2$ (buried to a $1.4 \text{ \AA}$ probe radius) on each molecule, a value consistent with the moderate affinity of the complex partners (Supplementary Movie S1). Two contiguous subsites that mainly encompass electrostatic interactions and hydrophobic/apolar contacts, respectively, are clearly identified (Figure 1B; Table I). In the first subsite, an electropositive patch formed by the Nr1β Arg109 and Arg232 side chains

Figure 1 The Nr1β–NL4 complex and binding interface at 2.6 Å resolution. (A) Overall structure of the Nr1β–NL4 complex, with a yellow NL4 dimer, magenta four-helix bundle, cyan Cys-loops, green Nr1β molecules and grey Ca^{2+} ; the N- and C-termini are labelled. (B) The amphiphilic character of the binding interface, most evident at the Nr1β surface with the contiguous blue electropositive and white hydrophobic/apolar patches, insures attraction and proper orientation of the partnering molecules. Electrostatic surface potentials of the Nr1β (top) and NL4 (bottom) molecules are contoured at $-0.3/+0.3 \text{ V}$, in which red describes negative and blue positive potentials. Both molecules are rotated 90° from (A) in opposite directions along the horizontal axis, and the molecular surface involved in the interaction is outlined in black. (C) The Ca^{2+} position and coordination in the Nr1β–NL4 (left) and Nr1β–NL1 (middle) complexes and the Nr1β LNS domain (3BOD; Koehnke *et al*, 2008) (right) are similar. The omit difference electron density map (blue) for the Ca^{2+} is contoured at 5σ in the Nr1β–NL4 complex. Water molecules were not visible in the structure of the Nr1β–NL1 complex because of its lower resolution (3.5 Å).

Table 1 Nrx1β and NL4 residues in contact, and NL1-specific contacts

Nrx1β	NL4 ^a	NL1 ^b
<i>Hydrogen bonds</i>		
Asn103	Arg561	
OD1 ^c	NH2 ^c	
O ^{d,e}	Asp366	
	OD1/OD2 ^{d,e}	
Ser107	Asn364	
NH ^d	OD1 ^d	
O		Lys306 NZ ^f
Arg109	Glu270^b	
NH ^e	OE2 ^e	
NH1	OE1	
NH1	Tyr365	
	OH ^e	
Arg232	Asp351	
NH1 ^c	OD1 ^c	
NH2	Gln359	
	OE1	
Thr235	Glu361	
NH	OE1 (d)/OE2(c)	
OG1	OE2	
Ile 236	Glu361	
NH ^c	OE1 ^c	
Asn238	Gly360	
ND2	O	
<i>Hydrophobic contacts^g</i>		
Asn103	Pro466	
Arg105 ^d	Asn364^d	
Pro106	Asn364	Lys306 ^h
Ser107	Asn364	
	Leu363	
Thr108	Leu363	
Arg109	His267	
	Glu270^b	
Leu135	Gln462	
	Tyr463	
Leu234	Gln359	
	Glu361	
Thr235	Glu361	
Ile236	Glu361	
	Phe362	
	Leu363	
Asn238	Tyr463	
Ser239	Tyr463	
	Gly464	

^aNL4 residues conserved among all neuroligins are labelled in bold.

^bContacts in the Nrx1β–NL4 complex are conserved in the Nrx1β–NL1 complex, except for those involving Glu270 (corresponding to Glu297 in NL1) because of the insertion of Lys306 in NL1.

^cContact observed only in subunit B of the dimer present in the asymmetric unit.

^dContact observed only in subunit A of the dimer present in the asymmetric unit.

^eInteraction through a water molecule.

^fContact observed only in subunits A and C of the two dimers present in the asymmetric unit.

^gWithin a 4.5 Å distance between carbon atoms from each partner in the complex.

^hContact observed only in subunits A and B of the two dimers present in the asymmetric unit.

and the Ca²⁺ nestled at the Nrx1β surface is complementary, in overall shape, dimensions and charge distribution, to the electronegative patch formed by the Glu270, Asp351 and Glu361 side chains at the NL4 surface. This subsite holds the Ca²⁺-mediated interactions and consists mainly of hydrogen-bonding interactions involving four water molecules. In turn, only two Nrx1β apolar side chains lie within

this positively charged subsite, Leu234 and Ile236, the latter having an important function for Nrx1β binding (see below). Hence, charge complementarity between the two partners highlights the crucial function of the Ca²⁺, whose coordination to negatively charged residues at the Nrx1β surface reinforces the electropositive motif and hence, not only prevents charge repulsion of the partners, but also favours their electrostatic attraction. *In the second subsite*, the binding interface mainly encompasses long-range hydrophobic/apolar contacts along with a few hydrogen bonds. Most of these contacts involve the peptidic backbone of each partner and small side chain residues, except for a hydrophobic stacking interaction between Nrx1β Leu135 and the phenol ring of NL4 Tyr463. Hence, the amphiphilic character of the complex interface likely accounts for the proper orientation of the two partnering proteins despite the limited contact area. Finally, three protruding Nrx1β side chains, Arg109 and Arg232 in the electropositive subsite and Asn103 at the edge of the hydrophobic/apolar subsite, are suitably positioned to serve as boundary clamp for stabilizing the complex, consistent with our earlier interpretation (Fabrichny *et al*, 2007).

Conformational rearrangements in NL4 associated with Nrx1β binding

Structural analysis of Nrx1β-bound versus unbound NL4 reveals conformational differences in two distinct regions at the NL4 surface. *The first region* corresponds to the Nrx1β-binding site, the topology of which is entirely reshaped through concerted positional rearrangement of several side chains to accommodate Nrx1β binding (Figure 2A; Supplementary Movie S2). Most notably, the side chains of Glu361 and Leu363 at the centre of the interface along with those of Glu270 and His267 on the one edge and Tyr463 on the other edge of the interface undergo marked swapping and/or rotation motions upon Nrx1β association (Supplementary Table S1). These conformational displacements disrupt the intricate tripartite hydrogen-bonding network formed by the side chains of Glu270 and Glu361, that form a glutamic acid bridge, and of His267, observed in unbound NL4. As a consequence, the Glu361 side chain is stabilized in a suitable position for Ca²⁺ coordination, whereas a cavity formed by Leu363, His267 and Glu270 is created to accommodate the guanidinium group in Arg109 of Nrx1β. Formation of a glutamic acid bridge between Glu270 and Glu361 in NL4 requires protonation of at least one of their two carboxyl groups. In fact, NL4 was crystallized at pH4.3, a value close to the glutamate pK_a value, but the Glu270–Glu361 interaction is likely to occur under physiological conditions as well. In unbound NL4, the side chain of His267 interacts with Glu270 and could locally modify its pK_a value, thus participating to stabilization of the Glu270–Glu361 bridge. In NL1, the corresponding His294 contributes to pH dependency of the Nrx1β–NL1 interaction (see below).

The second region where the topologies of Nrx1β-bound and unbound NL4 diverge significantly is located on the subunit face that is opposite to the Nrx1β-binding site, and where the active site gorge opens in AChE (Supplementary Figure S1). Here, the Cys-loop (Cys110–Cys146) adopts distinct conformations in the two unbound NL4 subunits, both differing from that observed in Nrx1β-bound NL4,

Figure 2 Conformational rearrangements of the Nrx1 β -binding site occur in NL4, but not in NL1 upon binding to Nrx1 β . **(A)** The Nrx1 β -binding site has a distinct conformation in Nrx1 β -bound NL4 (NL4 in yellow and Nrx1 β in green) compared with unbound NL4 (orange). Black arrows depict displacement of NL4 residues upon Nrx1 β binding. In unbound NL4, the tripartite interaction between His267, Glu270 and Glu361 prevents Nrx1 β binding and indirect Ca²⁺ coordination. In one subunit of the unbound NL4 dimer, His267 adopts an alternate conformation corresponding to that found in Nrx1 β -bound NL4 (not shown). **(B)** The Nrx1 β -binding site in unbound NL1 (blue) is similar to that in NL4 bound to Nrx1 β (both displayed as in **A**). Numbering in parentheses corresponds to NL1 residues. Owing to the insertion of Lys306 in NL1, the Nrx1 β -binding site in unbound NL1 is complementary for Nrx1 β binding and does not undergo conformational rearrangements upon complex formation.

whereas the neighbouring loops L3 (Gln477–Ser487) and L4 (Ile502–Ser513) display different conformations in the two structures. This observation further illustrates flexibility of the NL4 Cys-loop (Fabrichny *et al*, 2007) and highlights sequence variations associated with conformational mobility of the corresponding surface loops among members of the α/β -hydrolase-fold family. The function of these loops in neuroligins and the relevance of conformational rearrangements occurring on this face of the NL4 subunit upon Nrx1 β binding on the opposite face are unclear. Presence of a vestigial active centre cavity filled with water molecules (Fabrichny *et al*, 2007) may impart overall flexibility and a more distensible NL4, reflected in conformational changes of surface loops on the opposite side. These conformational rearrangements may also be associated with recognition and binding of a still non-identified second neuroligin partner.

Structural comparison of the Nrx1 β –NL4 and Nrx1 β –NL1 complexes

The extracellular domains of NL4 and NL1 (without splice inserts A and B) share ~80% of sequence identity and display very similar structures (see RMSD values in Materials and methods). All the residues forming the Nrx1 β -binding surface of NL4 are conserved in NL1, except for two conservative substitutions (Asn498 and Phe499 in NL1 for Gln462 and Tyr463 in NL4) that are most unlikely to alter the Nrx1 β interaction network. Indeed, neither the side chain of NL4 Gln462 nor that of NL1 Asn498 do interact with bound Nrx1 β , whereas NL4 Tyr463 interacts through its phenol ring, as does NL1 Phe499 (Araç *et al*, 2007), with no contribution of its hydroxyl group (Figure 2B). However, for NL1, the insertion of Lys306 between Glu297 and Gly307 in the absence of splice insert B (Supplementary Figure S2), found at the Nrx1 β -binding site, is a unique feature of this neuroligin subtype. In the Nrx1 β –NL1 complex, Lys306 interacts with Nrx1 β Pro106 and Ser107 (Table I), and pushes the adjacent Glu297 side chain towards NL1 Arg259 at the edge of the interface. In the Nrx1 β –NL4 complex, the corresponding negatively charged Glu270 residue faces Nrx1 β and interacts with its Arg109.

There is no conformational rearrangement of the Nrx1 β -binding site of NL1 upon Nrx1 β binding, as shown by perfect

superimposition of this region in the unbound and Nrx1 β -bound NL1 structures (Araç *et al*, 2007). In fact, as a result of the Lys306 insertion, the Glu297 position and stabilization through a salt bridge with Arg259 prevent any interaction with Glu397, contrary to that observed between the corresponding Glu270 and Glu361 residues in unbound NL4 (Figure 2A). Consequently, the binding site topology in unbound NL1 is preset for Nrx1 β binding. Indeed, the Glu397 side chain orientation is compatible with Ca²⁺ coordination and the cavity formed by His294 and Leu399 (but not Glu297, because of its interaction with Arg259) can accommodate Nrx1 β Arg109 (Figure 2B).

Characterization of Nrx1 β binding to neuroligins

The energetics and the dynamic properties of the interaction between Nrx1 β and NL4, NL1 or NL2 were characterized using SPR. Nrx1 β binding to immobilized NL4 and NL1 in physiological buffer conditions yielded sensorgrams whose squared profile denotes a labile interaction dictated by high association and dissociation rates (Figure 3A and B). Such a dynamic neuroligin–neuroligin interaction process, which possibly reflects a similar behaviour at the synapse, may also explain that more ordered crystals of the Nrx1 β –NL4 complex were obtained at 4°C than at 20°C, presumably by slowing down complex dissociation. The equilibrium dissociation constants determined for the NL4 and NL1 complexes (K_D values of 115 and 16 nM, respectively) are similar to those earlier reported (Comoletti *et al*, 2006; Araç *et al*, 2007). The lower affinity of Nrx1 β for NL4 compared with NL1 is consistent with the conformational rearrangements associated with Nrx1 β binding to NL4, that most likely occur at the expense of the energetics for complex formation.

The very weak Nrx1 β binding to NL2 did not allow us to calculate a dissociation constant, a limitation consistent with the low μ M-range value determined using a reverse ligand/analyte orientation (Comoletti *et al*, 2006). NL4 Gly464 is conserved in all neuroligins except NL2, in which it is replaced by Gln475 (Figure 4A). Comparative structural analysis indicates that the Gln substitution in NL2 should alter Nrx1 β binding through steric hindrance with the facing Nrx1 β Ser132 and Ser239 residues (Figure 4B). To ascertain the critical function of the side chain at this

Figure 3 SPR measurement of Nrx1 β binding to NL4 and NL1. Increasing concentrations of Nrx1 β and its mutants (as indicated) were injected over immobilized NL1 (**A**, **C**) and NL4 (**B**, **D**). Typical sensorgrams obtained with Nrx1 β are displayed (**A**, **B**). The measured plateau values were used to construct and calculate the adsorption isotherms (**C**, **D**). The Nrx1 β curves (black squares) correspond to the sensorgrams shown in (**A**, **B**). Those K_D values that could be calculated are indicated (ND, not determined). (**E**) Ionic strength and pH conditions of running and sample buffers were modified as indicated and the binding of Nrx1 β (320 nM) on immobilized NL1 or NL4 was measured. Binding obtained on NL1 at an ionic strength of 150 mM and pH 7.4 was set as 100% value for full occupation (asterisks). Values are means $\pm$ s.d. ($n = 3$). (**F**) Nrx1 β and its mutants (320 nM) were injected over NL1 and NL4 and the binding signal was measured. The signal obtained with Nrx1 β on NL1 was set as 100% value for full occupation (asterisk).

position, we Ala-substituted NL1 Gly500 that corresponds to NL4 Gly464 and NL2 Gln475, and assayed the Nrx1 β affinity of the mutant relative to that of NL1. As expected, addition of a methyl group at this position in NL1 totally abolished Nrx1 β binding. Conversely, a Gly substitution to NL2 Gln475 restored Nrx1 β binding to a level comparable with that of NL1. Hence, a single side chain substitution at this position in NL1 and NL2 is sufficient to promote or abolish Nrx1 β binding (Figure 4C).

Nrx1 β binding to NL1 and NL4 was also analysed as a function of ionic strength and pH (Figure 3E). When ionic strength is varied, Nrx1 β binding to NL1 is maximal between 75 and 150 mM NaCl, but it drops by $\sim 50\%$ at 300 mM NaCl as reported (Comoletti *et al*, 2003). Whereas Nrx1 β binding to NL1 and NL4 are similar at 75 mM NaCl, the binding to NL4 drops by $\sim 50\%$ at 150 mM NaCl and by $\sim 90\%$ at

300 mM NaCl. These data not only confirm that electrostatic interactions largely contribute to regulate the neuroligin-neurexin interaction, but they also emphasize a higher sensitivity of the Nrx1 β -NL4 complex to ionic strength variation, compared with the Nrx1 β -NL1 complex.

Nrx1 β binding to NL1 reaches a maximum between pH 8.5 and 7.4, but it falls by $\sim 60\%$ as the pH is lowered to 6.5. Nrx1 β binding to NL4 is also maximum at pH 8.5, but it falls by $\sim 50\%$ at pH 7.4 and by $>90\%$ at pH 6.5. The markedly higher sensitivity of the Nrx1 β -NL4 interaction to low pH compared with the Nrx1 β -NL1 interaction correlates with the conformational rearrangements observed in the Nrx1 β -binding site of NL4, but not that of NL1. In fact, the existence of the Glu270-Glu361 interaction in the absence of bound Nrx1 β and its strengthening at low pH are fully consistent with the marked weakening of the Nrx1 β -NL4 interaction when the

Figure 4 NL2 Gln475 is responsible for the low binding of Nrx1 β to NL2. (A) Sequence conservation between the Nrx1 β interacting loops in NL4 and the corresponding loops in NL1, NL2 and NL3. NL1 and NL4 numbering are displayed above and below the alignment, respectively. The position of NL2 Gln475 is indicated by a vertical arrow below the alignment. NL4 and NL2 residues also displayed in (B) are labelled in bold. (B) Superimposition of NL2 (cyan) and Nrx1 β -bound NL4 (yellow NL4 and green Nrx1 β). The substitution of NL4 Gly464 (yellow sphere, corresponding to NL1 Gly500) by Gln475 in NL2 (labelled in parenthesis) would result in a steric clash with Nrx1 β Ser132 and Ser239 at the interface. (C) SPR measurement of binding of NL1, NL2 and their respective G500A and Q475G mutants to Nrx1 β . Neuroligins (111 nM) were injected over immobilized Nrx1 β ; the signal obtained with NL1 was set as 100% occupation. Values are means $\pm$ s.d. ($n = 3$).

Figure 5 The Nrx1 β residues selected for mutagenesis study. (Bottom) Locations of Asn103, Arg232 and Ile236 are denoted at the Nrx1 β surface (green Nrx1 β molecule, with the molecular surface involved in the interaction in light green). The SS4 position and the Ca²⁺ are displayed as green and grey spheres, respectively. The fractional binding of each Ala-substituted mutant to NL1 and NL4 relative to that of Nrx1 β is indicated. (Top) Close-up view of the solvent and protein surface environments of selected Nrx1 β residues at the complex interface. For clarity purposes, different orientations were used in each panel. (A) Arg232 is located at the edge of the electropositive subsite of Nrx1 β . The omit difference electron density map (blue) for Nrx1 β Gly231–Leu234, NL4 Pro350–Asp352 and Glu358–Glu360 is contoured at 1 σ . (B) Arg109 is located in the electropositive subsite of Nrx1 β in a position central to the interface. Its side chain is buried in the cavity formed by NL4 His267, Glu270 and Leu363. (C) Ile236 is a hydrophobic/apolar residue located in the electropositive subsite of Nrx1 β and that coordinates the Ca²⁺ through its carbonyl. (D) Asn103 is located at the edge of the hydrophobic/apolar subsite of Nrx1 β and its protruding conformation is maintained by the presence of Pro101 and Pro102. The hydrogen-bonded interaction of Asn103 with NL4 Arg561 is not observed in the second Nrx1 β molecule bound to the NL4 dimer present in the asymmetric unit (cyan).

pH is lowered in the SPR assay. We suggest that His267 with a protonated imidazolium may facilitate the Glu270–Glu361 interaction in unbound NL4 through local modification

of the pK_a value of the Glu270 side chain (see above) (Figure 2A). In NL1, Ala substitution of the corresponding His294 strongly reduces the inhibition of Nrx1 β binding by

lowering the pH (Supplementary Figure S3). This result establishes that NL1 His294 has a critical function in the pH-dependent regulation of Nrx1 β –NL1 interaction and argues for involvement of the corresponding NL4 His267 in pH-related activity to control Nrx1 β binding as well.

Nrx1 β residues important for neuroligin binding

A similar structure-guided approach led us to substitute Ala residues for four interfacial Nrx1 β residues: Asn103, Arg109 and Arg232 that may serve as boundary clamp for NL4 binding and Ile236 that coordinates the Ca²⁺ and is one of the two hydrophobic/apolar residues present in the immediate environment of the cation (Figures 3C, D, F and 5). We also substituted Glu residues to Arg109 and Arg232 to create charge-reversal mutations. SPR analysis of the binding of these Nrx1 β mutants to immobilized NL1 and NL4 shows different characteristics of binding among the mutants, as well as differential electrostatic influence towards binding to the two neuroligin isoforms (Figure 3F).

An Ala substitution for Ile236 nearly abolishes Nrx1 β binding to NL1 and NL4 (>95% reduction) (Figure 5C). This substitution is not expected to abolish Ca²⁺ binding to Nrx1 β as the Ile236Ser mutant was found to retain Ca²⁺ binding (Reissner *et al*, 2008). Therefore, the Ile236 side chain is essential for binding to NL1 and NL4, by stabilizing the conformations of the facing NL4 Glu361 and Leu363 and/or by insuring the presence of a relatively hydrophobic/apolar environment near the Ca²⁺ to control surrounding solvation.

Substitution of Arg109 by Ala significantly reduces Nrx1 β binding to NL1 (by ~60%) and totally abolishes its binding to NL4 (Figure 5B), whereas the Arg109 substitution by Glu almost abolishes binding to NL1 (>95% reduction). These data, along with the structural interpretation that Nrx1 β Arg109 may contribute to the disruption of the tripartite His267–Glu270–Glu361 interaction present in unbound NL4, support a crucial function of Arg109 for both Nrx1 β recognition of and anchorage to NL4, and for its electrostatic guidance to NL1.

Substitution of Arg232 by Ala significantly reduces Nrx1 β binding to NL1 and NL4 (by ~70% and >90%, respectively), as does its substitution by Glu. Yet, in the electron density maps of the complex, no density is associated with the Arg232 side chain (Figure 5A), indicating high conformational flexibility of a side chain whose electrostatic contacts with the neuroligin partner are extremely weak and likely to be sensitive to ionic strength variations. Therefore, Arg232 seems to contribute to long-range attractive forces between neurexins and neuroligins through its positive charge and stabilization through its protruding and flexible side chain.

Substitution of Asn103 by Ala does not alter Nrx1 β binding to either NL1 or NL4. Hence, the protruding Asn103 side chain (Figure 5D), instead of participating to direct stabilization of the complex, could regulate complex formation through steric hindrance in preventing close contacts between the partners, resulting in the rapid dissociation (high k_{off} value) observed on the sensorgrams.

Binding of the four Nrx1 β Ala mutants to NL1 and NL4 displayed a behaviour similar to that of Nrx1 β in their binding sensitivity to ionic strength and pH variations, thereby confirming the higher sensitivity of Nrx1 β binding to NL4 compared with NL1 to these two parameters (data not shown).

Concluding remarks

The 2.6 Å-resolution structure of the Nrx1 β –NL4 complex reveals concerted conformational rearrangements at opposite surfaces of the NL4 subunit associated with Nrx1 β binding. Hence, this protein seems to be structured to show allosteric behaviour occurring at distant sites. Those rearrangements that cooperate to reshape the Nrx1 β -binding site could not have been predicted by molecular modelling; yet, they are largely corroborated by mutagenesis and SPR data. Whether Nrx1 β binding induces a transition to a new conformational state of NL4 or stabilizes one among a population of transient conformations in solution is not clear. We propose that these rearrangements account for conformationally linked regulation of the Nrx1 β –NL4 interaction, leading to a lower affinity and a higher sensitivity to ionic strength and pH variations compared with the Nrx1 β –NL1 association. Hence, they would contribute to a greater range of recognition capacities for the neurexins than would be achieved with a population of static adhesion molecules. As rearrangements also involve the NL4 face opposite to the Nrx1 β -binding site, it is tempting to consider that the prominent gorge in the cholinesterases that is capped in the neuroligins (Fabrichny *et al*, 2007) may provide the structural basis for the overall flexibility of the molecule. Rearrangement of this face of the NL4 molecule might be associated with the recognition of a second partner, distinct from Nrx1 β , as earlier hypothesized from surface charge distribution analysis (Fabrichny *et al*, 2007).

The physiological relevance of this conformational reshaping unique to NL4 remains unknown. Despite its low abundance in brain, NL4 has a crucial function in brain functioning, as inferred from its genotype linkage to autism spectrum disorders (Jamain *et al*, 2008). Yet, contrary to the other neuroligins, the synaptic function of NL4 is not regulated by the presence/absence of alternatively spliced inserts. Macroscopic changes of physico-chemical conditions in the brain are tightly controlled to insure homeostasis, but local pH fluctuations occurring in specific microdomains, such as synapses, might be significant (Waldmann *et al*, 1997; Wemmie *et al*, 2002). Synaptic vesicles seem to be acidic (pH ~5.7) (Miesenböck *et al*, 1998) and their release may cause transient acidification of the synaptic cleft accompanying synaptic activity (Krishtal *et al*, 1987; Chesler and Kaila, 1992). These rapid and localized pH changes in the vicinity of the neuronal membrane are likely to be more pronounced than the macroscopic pH fluctuations measured, for example in hippocampal slices (Krishtal *et al*, 1987), and could locally influence Nrx1 β –NL4 interaction. Hence, the NL4-specific conformational rearrangements observed in our study could be associated with fine tuning of the Nrx1 β –NL4 interaction through environmental variations occurring during synapse formation.

Materials and methods

Protein expression and purification

The soluble AChE-like domains of human NL4 (residues Gln44–Thr619), flanked with an N-terminal FLAG epitope and an intervening linker numbered (-12)DYKDDDDKLAAA(-1), and those of rat NL1 (residues Gln46–Asp638, without inserts at positions SSA and SSB) and NL2 (residues Pro65–Leu615, without an insert at position SSA), were expressed from stable HEK293 cells and purified from the culture medium on immobilized anti-FLAG M2 antibody using, as the eluent, 100 µg/ml FLAG peptide in 10 mM

Hepes pH 7.4, 150 mM NaCl, 1 µg/ml leupeptin (Comoletti *et al*, 2003). NL4 was further purified by gel-filtration FPLC on prepacked Superdex-200 (GE Healthcare) equilibrated and eluted with 10 mM Na-HEPES pH 7.4, 50 mM NaCl, 0.01% NaN₃ (buffer A). The soluble LNS domain of rat Nrx1β (residues Gly81–Val288, without an insert at position SS4 and the Nrxβ-specific N-terminal sequence), flanked with a N-terminal 6xHis tag upstream of a TEV cleavage site, was expressed from Rosetta PlyS cells (Fabrichny *et al*, 2007). Nrx1β was purified by immobilized-nickel affinity FPLC on a HisTrap-FF Crude cartridge (GE Healthcare). Tag removal used a 6xHis-tagged TEV protease (Invitrogen), a second nickel affinity chromatography and an ultimate gel filtration on prepacked Superdex-200 in buffer A. Site-directed mutagenesis of the NL1, NL2 and Nrx1β cDNAs used the QuikChangeII kit (Stratagene); the mutants were expressed and purified using the same protocols as described above. The purity and molecular masses of the purified proteins were controlled by SDS–PAGE (neuroligins and neuexins) and MALDI-TOF spectrometry (neuexins). Proper folding of the neuroligin mutants was assessed from their high level of expression in the cell culture medium (Sitia and Braakman, 2003) and unaltered solubility during purification. Proper folding of the Nrx1β mutants was assessed from their chromatographic behaviour and a fluorescence-based thermal denaturation assay (Supplementary Figure S4).

The Nrx1β–NL4 complex was formed in solution using a 1.2 molar excess of Nrx1β in 10 mM HEPES pH 7.4, 100 mM NaCl, 2 mM CaCl₂ (ON incubation, 4°C). The buffer composition and final complex concentration (8.0 mg/ml) were adjusted by ultrafiltration and UV spectrophotometry.

Crystallization, data collection and processing

The Nrx1β–NL4 complex was crystallized at 4°C by vapour diffusion using a protein-to-well ratio of 0.5:1 (v/v) in 1.5 µl hanging drops and 10% PEG20000, 100 mM MES pH 6.3, 2 mM CaCl₂ as the well solution. Crystals were briefly transferred to the well solution supplemented with 30% (v/v) ethylene glycol and flash cooled in liquid nitrogen. X-ray diffraction data up to 2.6 Å resolution were collected on beamline ID23-EH1 at the ESRF. The data were indexed and integrated with the program MOSFLM (Leslie, 1992) and merged and scaled with SCALA (CCP4, 1994). Data collection statistics are reported in Table II. The asymmetric unit contains an NL4 dimer bound with two Nrx1β molecules. Compared with earlier crystals grown at 20°C and diffracting to 3.9 Å (Fabrichny *et al*, 2007), the new crystals grown at 4°C retain the orthorhombic space group, P2₁2₁2, but their more compact unit cell, dictated by a ~15% shorter cell parameter *a*, likely contributes to the higher resolution.

Structure solution and refinement

The structure of the Nrx1β–NL4 complex was solved by molecular replacement using MOLREP (Vagin and Teplyakov, 1997) and, as a template, the initial structure of the same complex (accession code 2VH8). The model was refined with Refmac5 (Murshudov *et al*, 1997), including tight NCS restraints for the two NL4 molecules (excluding the Cys-loop Pro111–Gln142 and loops Pro63–Gly68 and Gly408–Lys413) and the two Nrx1β molecules, and TLS refinement. TLS groups generated using the TLS Motion Determination server (Painter and Merritt, 2006) were manually adjusted to five groups for each NL4 molecule (Ala43–Cys110/Asn143–Tyr291/Phe340–Val373/Trp449–Phe472/Arg561–Tyr582, Pro111–Gln142, Gln292–Ala339, Gly374–Gln448/Arg583–His598 and Tyr473–Ser560), and two groups for each Nrx1β molecule (His82–Gly94/Gly256–Val288 and Gln95–Gln255). The structure was manually corrected using COOT (Emsley and Cowtan, 2004).

The final model comprises the FLAG residue Ala(-1) and NL4 residues Gln44–Leu62, Leu67–Glu156, Ser164–Val540, Val557–His598 for one subunit and the FLAG residues Lys(-10)–Ala(-1) and NL4 residues Gln44–Glu156, Lys165–Val540, Val557–His598 for the second subunit; Nrx1β residues His82–Val288 in both subunits; five chloride ions, one MES molecule and 30 ethylene glycol molecules arising from the crystallization and cryoprotectant solutions, respectively. GlcNac moieties are linked to Asn102 and Asn511 in both NL4 subunits. Missing electron densities correspond to NL4 surface loops Pro63–Ile66, Asp157–Asn164 and Pro541–Glu556. Data collection and refinement statistics are reported in Table II.

Table II X-ray data collection and refinement statistics for the Nrx1β–NL4 complex

<i>Data collection</i>	
Wavelength (Å)	0.9834
Space group	P2 ₁ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	136.30, 198.77, 85.89
Resolution (Å) ^a	30–2.6 (2.74–2.6)
Unique reflections ^a	72 526 (10 456)
Redundancy ^a	4.7 (4.8)
Completeness (%) ^a	99.9 (100.0)
<i>I</i> /σ ^a	10.7 (2.8)
<i>R</i> _{merge} (%) ^{a,b}	10.0 (55.4)
<i>Refinement and model quality</i>	
Resolution (Å)	30–2.6
Reflections	68 827
<i>R</i> _{cryst} / <i>R</i> _{free} (%) ^c	19.0/22.2
Number of atoms	11 720
Protein	11 176
Ligand/sugar/ion	132/56/7
Water	349
<i>Average B-factors (Å²)</i>	
Protein	44.5
Ligand/sugar/ion	65.1/83.3/57.0
Water	25.3
<i>RMSD^d</i>	
Bond (Å)	0.015
Angle (deg)	1.55
<i>Ramachandran plot (%)</i>	
Most favoured regions	85.6
Additionally allowed regions	14.3
PDB accession code	2XB6

^aValues in parentheses are for the highest-resolution shell.

^b $R_{\text{merge}} = \sum_{\text{hkl}} (\sum_i |I_{\text{hkl}} - \langle I_{\text{hkl}} \rangle|) / \sum_{\text{hkl}} \langle I_{\text{hkl}} \rangle$.

^c $R_{\text{cryst}} = \sum_{\text{hkl}} \|F_o\| - \|F_c\| / \sum_{\text{hkl}} \|F_o\|$; R_{free} is calculated for 5% of randomly selected reflections excluded from refinement.

^dRMSD from ideal values.

Structural analysis

The RMSD between the two NL4 subunits, calculated by LSQMAN (Kleywegt, 1996), is 0.17 Å for 528 Cα atoms. As the NL1 molecule in structure 3B3Q (Chen *et al*, 2008) does not contain Lys306, for our comparison we used the Lys306-containing NL1 structures 3BIX and 3BIW, respectively unbound and bound to Nrx1β (Araç *et al*, 2007). Superimposition of each of the two Nrx1β-bound NL4 subunits present in our structure with each of the four Nrx1β-bound NL1 subunits present in structure 3BIW resulted in an average RMSD of 0.72 Å for at least 518 Cα atoms.

The NL4 and Nrx1β electrostatic surface potentials shown in Figure 1 were calculated using CCP4mg (Potterton *et al*, 2002). Figures were prepared with PyMOL (<http://www.pymol.org>).

Further refinement of the low-resolution structure of the Nrx1β–NL4 complex

The initial, 3.9 Å-resolution structure of the Nrx1β–NL4 complex, solved using the structures of the NL4 dimer (3BE8; Fabrichny *et al*, 2007) and of Nrx1β (1C4R; Rudenko *et al*, 1999) as templates, had been refined with final *R*_{fac}/*R*_{free} values of 23.1/32.3% associated with the presence of poorly defined regions in the electron density maps (Fabrichny *et al*, 2007). The strong model bias arising from the molecular replacement solution and the limited resolution of the data precluded revealing NL4 conformational rearrangements upon Nrx1β binding. To reduce model bias, the present 2.6 Å-resolution structure of the Nrx1β–NL4 complex was used as a new template to correct uncertainties within two regions of functional relevance: the Cys-loop region and the Nrx1β-binding site. This procedure yielded *R*_{fac}/*R*_{free} values of 20.5/27.5%, significantly lower than the initial values (Supplementary Table S2). The coordinates of this alternatively refined structure have been deposited with the Protein Data Bank (accession code 2WQZ).

SPR analysis of neuroligin-neurexin interactions

SPR experiments were performed with a Biacore 3000 apparatus (GE Healthcare) at 25°C. For data shown in Figure 3, 60–120 fmol of purified NL4, NL1 and NL2 were covalently immobilized on a CM5 sensor chip using amine-coupling chemistry according to the manufacturer's instructions. Purified Nrx1 β and the four mutants were injected at a flow rate of 20 μ l/min for 3 min using 10 mM HEPES pH 7.4, 150 mM NaCl, 2 mM Ca²⁺ as the running buffer (except where otherwise noted). After recording of spontaneous dissociation, the sensor chip was regenerated with 5 mM EDTA for 15 s. Sensorgrams obtained from the fourth, empty flow cell were systematically subtracted from those obtained with the neuroligin cells. The K_D values for Nrx1 β binding to the immobilized neuroligins were calculated by plotting saturation-binding curves, using the equilibrium response at the plateau of all curves, versus Nrx1 β concentrations. For data shown in Figure 4, a reverse ligand/analyte orientation was used: 25–50 fmol of Nrx1 β were covalently immobilized on the chip and NL1, NL2 and their respective mutants were injected at a flow rate of 50 μ l/min for 5 min using the running buffer complemented with 0.005% (v/v) P20. After each experiment, the chip was regenerated with 1 M NaCl, 5 mM EGTA, 0.005% P20 for 30 s.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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