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Neuroligin-1 performs neurexin-dependent and neurexin-independent functions in synapse validation

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Postsynaptic neuroligins are thought to perform essential functions in synapse validation and synaptic transmission by binding to, and dimerizing, presynaptic α- and β-neurexins. To test this hypothesis, we examined the functional effects of neuroligin-1 mutations that impair only α-neurexin binding, block both α- and β-neurexin binding, or abolish neuroligin-1 dimerization. Abolishing α-neurexin binding abrogated neuroligin-induced generation of neuronal synapses onto transfected non-neuronal cells in the so-called artificial synapse-formation assay, even though β-neurexin binding was retained. Thus, in this assay, neuroligin-1 induces apparent synapse formation by binding to presynaptic α-neurexins. In transfected neurons, however, neither α- nor β-neurexin binding was essential for the ability of postsynaptic neuroligin-1 to dramatically increase synapse density, suggesting a neurexin-independent mechanism of synapse formation. Moreover, neuroligin-1 dimerization was not required for either the non-neuronal or the neuronal synapse-formation assay. Nevertheless, both α-neurexin binding and neuroligin-1 dimerization were essential for the increase in apparent synapse size that is induced by neuroligin-1 in transfected neurons. Thus, neuroligin-1 performs diverse synaptic functions by mechanisms that include as essential components of α-neurexin binding and neuroligin dimerization, but extend beyond these activities.

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Introduction

Synapses are specialized intercellular junctions that allow neurons to communicate rapidly and precisely with each other. It is likely that multiple cell-adhesion molecules connect pre- and postsynaptic neurons, but only few genuinely synaptic cell-adhesion molecules have been described. These include neuroligins and neurexins, a pair of heterophilic cell-adhesion molecules that are present in all neurons (Ushkaryov *et al*, 1992; Ichtchenko *et al*, 1995; Ullrich *et al*, 1995).

Mammals express four principal neuroligins (neuroligin-1 to -4; Ichtchenko *et al*, 1996; Bolliger *et al*, 2001, 2008) and six principal neurexins (neurexin-1α to -3α and -1β to -3β; Ushkaryov and Südhof, 1993; Ushkaryov *et al*, 1994; Rowen *et al*, 2002; Tabuchi and Südhof, 2002). All neuroligins are composed of a single large extracellular domain homologous to esterases (but without catalytic activity), a single transmembrane region, and a short cytoplasmic tail. The esterase domain constitutively dimerizes (Comoletti *et al*, 2003).

Neurexins are expressed as α- and β-neurexins from three genes (neurexin-1α and -1β to -3α and 3β). α-Neurexins are composed of multiple extracellular domains (six LNS (lamin, neurexin, sex hormone-binding protein)- and three EGF-domains), whereas β-neurexins contain only a single extracellular LNS-domain; in both α- and β-neurexins, the LNS-domains are followed by a single transmembrane region and a short cytoplasmic tail.

Neurexins are extensively alternatively spliced, creating hundreds of variants (Ullrich *et al*, 1995), whereas neuroligins are alternatively spliced at only two positions (Boucard *et al*, 2005). Neuroligins and neurexins bind to each other in a tight complex with nanomolar affinity that is held together by central Ca^{2+}-ions (Arac *et al*, 2007; Fabrichny *et al*, 2007; Chen *et al*, 2008). This complex exhibits different affinities depending on the isoform and splice variants of neurexins and neuroligins involved (Boucard *et al*, 2005; Chih *et al*, 2006; Comoletti *et al*, 2006). At least neuroligin-1 and -2 are differentially localized, with neuroligin-1 being exclusively present in excitatory synapses and neuroligin-2 in inhibitory synapses (Song *et al*, 1999; Graf *et al*, 2004; Varoqueaux *et al*, 2004; Levinson *et al*, 2005).

Extensive studies showed that neuroligins act as synaptic cell-adhesion molecules, but the precise nature of their function remains unclear (e.g. see Baksh *et al*, 2005; Chubykin *et al*, 2007; Conroy *et al*, 2007; Dong *et al*, 2007; Futai *et al*, 2007). An initial pioneering study on neuroligin-1 (Scheiffele *et al*, 2000), subsequently confirmed for other neuroligins and extended to neurexins (Graf *et al*, 2004; Chubykin *et al*, 2005; Nam and Chen, 2005), showed that when neuroligin-1 is expressed in a non-neuronal cell that is then co-cultured with primary neurons, these neurons form synapses onto the non-neuronal cell. This experiment, referred to as the artificial synapse-formation assay, led to

the notion that neuroligins and neurexins may function in synapse formation, and that they act by binding to each other (Cline, 2005; Dean and Dresbach, 2006; Lisé and El-Husseini, 2006; Gottmann, 2008). Consistent with this notion, neurexins are enriched in presynaptic nerve terminals contacting a neuroligin-expressing non-neuronal cell (Chubykin *et al*, 2005; Berninghausen *et al*, 2007).

In a second related assay, overexpression of neuroligins in transfected neurons increases the number of synapses onto these neurons, apparently confirming a synaptogenic activity for neuroligins (Chih *et al*, 2004; Boucard *et al*, 2005). Expression of neuroligin-1 in this experiment also leads to an increase in apparent synapse size, suggesting that neuroligin-1 not only increases synapse numbers, but also changes synapse properties (Boucard *et al*, 2005). As neuroligins form constitutive dimers (Comoletti *et al*, 2003), and many cell-surface receptors are activated by dimerization (Klemm *et al*, 1998), postsynaptic neuroligins were proposed to generally activate presynaptic neurexins by dimerizing them. In support of this notion, antibody-mediated dimerization of overexpressed neurexin in a neuron seems to induce local formation of presynaptic specializations (Dean *et al*, 2003).

Consistent with a synaptic function of neuroligins and neurexins, knockout studies revealed that deletions of neuroligins or neurexins in mice produce profound impairments of synaptic transmission (Missler *et al*, 2003; Kattenstroth *et al*, 2004; Varoqueaux *et al*, 2006; Chubykin *et al*, 2007). These results unequivocally established that neuroligins and neurexins perform central functions in synapses required for survival. However, in these studies, deletion of neuroligins or neurexins did not cause major decreases in synapse numbers, suggesting that these molecules function not in the initial induction of synapse formation, but in their functional organization, a process referred to as synapse validation (Chubykin *et al*, 2007). Consistent with this hypothesis, increases in synapse numbers in transfected neurons expressing neuroligins require synaptic activity (i.e. are not simply the consequence of passive cell adhesion, but the result of synaptic signalling, Chubykin *et al*, 2007). In agreement with their localizations to excitatory and inhibitory synapses, respectively, overexpression of neuroligin-1 only boosts excitatory synaptic inputs onto a neuron, whereas neuroligin-2 only enhances inhibitory inputs (Chubykin *et al*, 2007). Nevertheless, these results were puzzling because neuroligin-1 and -2 both bind to neurexins. Their neurexin binding exhibits different affinities (Boucard *et al*, 2005; Chih *et al*, 2006; Comoletti *et al*, 2006; Arac *et al*, 2007), but it is unclear how differences in neurexin-binding affinity could produce the diametrically different effects of neuroligin-1 and -2 on synaptic transmission.

Indeed, the function of neurexin binding in neuroligin function has not yet been examined directly. This issue is not only important for understanding how synaptic cell-adhesion shapes synaptic transmission in brain, but is also relevant for insight into cognitive diseases such as autism, schizophrenia, and drug addiction because neurexin and/or neuroligin genes have been linked to such diseases in human beings (reviewed in Südhof, 2008). Thus, to address this issue, we have now examined the importance of neurexin binding for the action of neuroligin-1 in synapse formation and synaptic transmission.

Results

Generation of neuroligin-1 mutants that do not bind to neurexins or do not dimerize

Using the atomic structure of the neuroligin-1/neurexin-1β complex as a guide (Arac *et al*, 2007), we designed a series of mutations in neuroligin-1 predicted to either alter neurexin binding to neuroligin-1, to abolish dimerization of neuroligin-1, or to change the surface of neuroligin-1 outside of its neurexin-binding and dimerization interfaces. In designing all of these mutants, we tried to avoid any interference with the folding of neuroligin-1. These mutants were produced in reiterative steps because the first round of mutagenesis (NL1–1 to 1–25) yielded only one mutant that lacked neurexin-1α binding, but still bound neurexin-1β (NL1–5; Table I), leading us to a second round of mutagenesis (NL1–31 to 1–46) that included the mutations of the NL1–5 mutant (Arac *et al*, 2007). All neuroligin-1 mutants analysed were expressed as fusion proteins with the monomeric green fluorescent protein mVenus, which was inserted into the cytoplasmic tail of neuroligin-1 always in the same position to allow neuroligin-1 detection independent of an intrinsic epitope.

We first examined the expression levels and surface transport of all neuroligin-1 mutants. These measurements were intended to eliminate misfolded neuroligin-1 mutants that are likely to be poorly expressed, and unlikely to reach the cell surface (Comoletti *et al*, 2004; De Jaco *et al*, 2006). Moreover, any neuroligin-1 mutant that does not reach the cell surface could not be functionally examined, and thus would need to be excluded from the analysis.

To measure surface transport of neuroligin-1 mutants, we expressed wild-type and mutant neuroligin-1 in transfected HEK293T cells. We then stained the transfected cells without detergent permeabilization with a monoclonal neuroligin-1 antibody (4C12) that reacts with the extracellular sequences of neuroligin-1 (Song *et al*, 1999), quantitated the amount of surface-exposed neuroligin-1 by indirect immunofluoresence, and measured the amount of total neuroligin-1 through its mVenus signal (Figure 1A; Supplementary Figures S1 and S2). Of 36 mutants tested, we found that 27 mutants expressed well, and correctly reached the cell surface, suggesting that these mutants are well folded (Table I; Figure 1; Supplementary Figures S1 and S2). These mutants include multiple neurexin-binding and dimerization mutants and were partly described earlier (Arac *et al*, 2007). To confirm the surface exposure of three key mutants (or lack thereof), we also performed surface biotinylation assays with similar results (Figure 1B; Supplementary Figure S3).

We next measured neurexin binding to all of the presumptively well-folded neuroligin-1 mutants, using surface binding of recombinant neurexin-1α and -1β to the transfected HEK293 cells (Figure 1C and D; Supplementary Figures S4 and S5). These experiments showed that among the initial mutants, only one mutant (NL1–5) was defective in neurexin-1α binding, but still bound neurexin-1β (Table I; Arac *et al*, 2007). Thus, in a second round of mutagenesis, we tested seven additional mutants that included all of the substitutions present in NL1–5 (Table I; Figure 1C and D; Supplementary Figures S4 and S5). Three mutants were found not to bind either α- or β-neurexins (NL1–32, –35, and –37; note that NL1–32 was extensively characterized biophysically earlier; Arac *et al*, 2007).

Table I Properties of neuroligin-1 mutants

Mutant name	Mutations (all in NL1 lacking inserts in splice sites A and B	Localization	α/β-Neurexin binding		Binding affinity (K_d)	Assays employed	Reference
			α	β			
NL1 WT	Wild type	PM	+	+	20.9 nM	ITC/SPR/SBA	Arac *et al* (2007)
NL1–1	D242N/Q243A/K246A	ER	ND	ND	ND	SBA	This study
NL1–2	Q256A/R259A	ER	ND	ND	ND	SBA	This study
NL1–3	Q328A/Y332A	PM	+	+	ND	SBA	This study
NL1–4	R371A/Y372A/H373A	PM	+	+	ND	SBA	This study
NL1–5	L399A/N400A/D402N	PM	–	+	>10 μM	SBA	Arac *et al* (2007)
NL1–6	D421N/D423N/D424N	PM	+	+	ND	SBA	This study
NL1–7	D440N/N441A/D424N	PM	+	+	ND	SBA	This study
NL1–8	N468A/E470A/R472A/K474A	ER	ND	ND	ND	SBA	This study
NL1–9	E542Q/L543A/F544A	ER	ND	ND	ND	SBA	This study
NL1–10	F582A/K586A/R589A	PM	+	+	ND	SBA	This study
NL1–11	E128Q/V129A	PM	+	+	ND	SBA	This study
NL1–21	E72Q/I73A	PM	+	+	ND	SBA	This study
NL1–22	D140N	PM	+	+	ND	SBA	This study
NL1–23	H294A/E297A	ER	ND	ND	ND	SBA	This study
NL1–24	L302A/S321A/S322A	ER	ND	ND	ND	SBA	This study
NL1–25	Y509A/H511A/Q513A	PM	+	+	ND	SBA	This study
NL1–31	L399A/N400A/D402N/K306A	PM	–	+	ND	SBA	This study
NL1–32	L399A/N400A/D402N/E297A/K306A	PM	–	–	>10 μM	SPR/SBA	Arac *et al* (2007)
NL1–33	L399A/N400A/D402N/L273A	PM	–	+	ND.	SBA	This study
NL1–34	L399A/N400A/D402N/K601A/D602A	PM	–	+	ND	SBA	This study
NL1–35	L399A/N400A/D402N/Q395A/E397A	PM	–	–	>50 μM	SPR/SBA	Arac *et al* (2007)
NL1–36	L399A/N400A/D402N/D571A/Q574A	ER	ND	ND	ND	SBA	This study
NL1–37	L399A/N400A/D402N/F499A	PM	–	–	ND	SBA	This study
NL1–38	T111A/Q112A	ER	ND	ND	ND	SBA	This study
NL1–39	E92A/H93A	PM	+	+	ND	SBA	This study
NL1–40	F458A/W463A	PM	+	+	ND	SBA	This study
NL1–41	Q147A/D148A	PM	+	+	ND	SBA	This study
NL1–42	R63A/K66A	ER	ND	ND	ND	SBA	This study
NL1–43	S102A/S105A	PM	+	+	ND	SBA	This study
NL1–44	F582A/K586A/R589A/E72Q/I73A	PM	+	+	ND	SBA	This study
NL1–45	Y509A/H511A/Q513A/D421N/D423N/D424N	PM	+	+	ND	SBA	This study
NL1–46	Q328A/Y332A/D440N/N441A/Y445A	PM	+	+	ND	SBA	This study
NL1–51	F458A/M459A/W463A	PM	+	+	ND	SBA	This study
NL1–53	F458A/M459A/W463A L629A/L633A	PM	+	+	ND	SBA	This study
NL1–54	F458A/M459A/W463A/L399A/N400A/ D402N/E297A/K306A	ER	ND	ND	ND	ND	This study
NL1–ΔC	T776 followed by stop codon	PM	+	+	ND	SBA	This study

ER, endoplasmic reticulum; ITC, isothermal titration calorimetry; ND, not determined; NL1, neuroligin-1; PM, plasma membrane; SPR, surface plasmon resonance; SBA, surface binding assay (Figures 1, Supplementary Figure S4 and S5); all neuroligin-1 molecules used lacked inserts in splice sites A and B, and contained a cytoplasmic monomeric mVenus tag inserted between residues 776 and 777.

We also performed a biophysical characterization of apparently well-folded dimerization mutants (NL1–40 and 1–51) to confirm that the mutation did indeed abolish dimerization. These mutants behaved as monomeric proteins during gel filtration and multi-angle laser light scattering and exhibited a normal circular dichroism spectrum, confirming that they are well folded, but monomeric (Figure 2). Thus, monomeric neuroligin-1 can reach the cell surface, arguing against the notion that neuroligin-1 dimerization is required for the export of neuroligin-1 out of the endoplasmic reticulum (Comoletti *et al*, 2003). Together, the 27 mutants that are transported to the cell surface and exhibit either a defect in neurexin binding, or in dimerization, or no apparent defect provide us with a toolkit to explore the function of neurexin binding and dimerization by neuroligin-1 in the synaptic activities of neuroligin-1.

α-Neurexin binding but not dimerization is essential for artificial synapse formation induced by neuroligin-1

A striking property of neuroligin-1 is its potent activity in the artificial synapse-formation assay (Scheiffele *et al*, 2000). In this assay, a non-neuronal cell is transfected with neuroligin-1 or a control protein, co-cultured with primary neurons, and the number of synapses formed onto the transfected non-neuronal cell by the co-cultured neurons is measured by immunofluorescence (Scheiffele *et al*, 2000; Biederer *et al*, 2002). We tested all neuroligin-1 mutants in this assay using transfected COS cells, and quantified the expression of mutant neuroligin-1 through its mVenus moiety and the amount of synapse formation on the transfected COS cells through indirect immunofluorescence for the presynaptic marker protein synapsin (Figure 3). All neuroligin-1 mutants that were well expressed on the cell surface and bound neurexins were active in the artificial synapse-formation assay. Strikingly, however, all neuroligin-1 mutants that exhibited no α-neurexin binding, independent of whether they still bound β-neurexins or not, were unable to induce synapse formation in this assay (Figure 3; Supplementary Figures S4 and S5). In contrast, the dimerization-deficient neuroligin-1 mutants (NL1–40 and 1–51) were fully active. These data show that in the artificial synapse-formation assay, neuroligin-1 acts by binding to presynaptic α-neurexins in the co-cultured neurons.

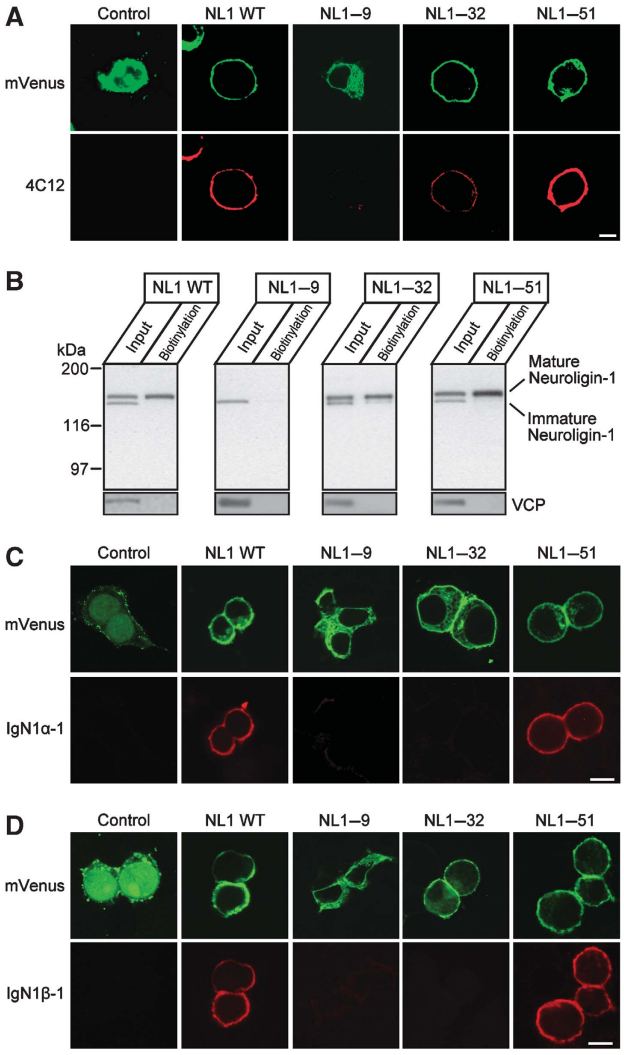

Figure 1 Analysis of the expression, surface transport, and α- and β-neurexin-binding properties of neuroligin-1 mutations. Panels depict representative data for wild-type neuroligin-1 (NL1 WT) and three key neuroligin-1 mutants that are either retained in the endoplasmic reticulum (NL1–9) or transported to the cell surface, but do not bind α- and β-neurexins (NL1–32) or do not dimerize (NL1–51). For complete datasets of all 37 mutants, see Supplementary Figures S1–S5 and Table I. In this and all following figures, all neuroligin-1 proteins are expressed as mVenus-fusion proteins; Control = mVenus alone. (**A**) Representative images of transfected HEK293T cells to illustrate the expression and surface transport of neuroligin-1 proteins. Transfected cells were fixed, but not permeabilized, and incubated with neuroligin-1 4C12 monoclonal antibody (raised against the extracellular region of neuroligin-1; Song *et al*, 1999). Total neuroligin-1 was visualized through its mVenus fluorescence (green), and cell-surface exposed neuroligin-1 by indirect immunofluorescence for the 4C12 antibody (red). Scale bar = 4 μm (applies to all images). For quantitations, see Supplementary Figure S2. (**B**) Immunoblot analysis of neuroligin-1 surface biotinylation. Surface proteins in transfected HEK293T cells expressing neuroligin-1 proteins were biotinylated and affinity purified on immobilized avidin. The input fraction and the biotinylated proteins were analysed by immunoblotting with antibodies to neuroligin-1 (4C12; top) and VCP (valosin-containing protein, bottom; used as a loading control). Note that only fully glycosylated, mature neuroligin-1 (upper band) is biotinylated. (**C**, **D**) Binding of Ig-fusion proteins of neurexin-1α (IgN1α-1; C) or -1β (IgN1β-1; D) to transfected HEK293T cells expressing neuroligin-1 proteins. Non-permeabilized transfected cells were incubated with 0.15 μM Ig-fusion proteins, analysed by fluorescence microscopy to visualize total expressed neuroligin-1 (through its mVenus fluorescence; green), and bound by Ig-fusion proteins (through antibodies to human IgG; red); scale bar = 4 μm, applies to all panels.

α- and/or β-neurexin binding are not required for the ability of neuroligin-1 to increase synapse density in neurons

Neuroligin-1 overexpression in transfected neurons induces a large increase (>50%) in the density of synapses onto that neuron (Chih *et al*, 2004; Boucard *et al*, 2005). This activity of neuroligin-1 was thought to reflect the same synaptogenic function as its activity in the artificial synapse-formation assay. However, testing the various neuroligin-1 mutants in transfected neurons yielded an unexpected result: neuronal overexpression of neurexin-binding-deficient neuroligin-1 mutants increased synapse density on these neurons to the same extent as overexpression of wild-type neuroligin-1, even when both α- and β-neurexin binding were blocked (Figure 4; Supplementary Figure S6). The same effect was observed for the dimerization-defective neuroligin-1 mutant NL1–51 (Figure 4). Thus, neurexin binding or dimerization are not required for neuroligin-1 to increase the density of synapses on a neuron, and the two different synapse-formation assays—the artificial synapse-formation assay based on neuroligin-1 expression in non-neuronal cells and the over-expression assay in neurons—measure distinct processes.

Besides increasing the density of synapses on a transfected neuron, overexpressed neuroligin-1 lacking an insert in splice site B also increases the apparent synapse size on these neurons (Boucard *et al*, 2005). This effect manifests as a significant increase of the signal for the postsynaptic marker, and an even bigger increase of the signal for the presynaptic marker, of the synapses contacting the transfected neuron, and thus must represent a trans-synaptic effect. Strikingly, when we studied the strength of the presynaptic synapsin signal in transfected neurons expressing various neuroligin-1 mutants, we found that all neurexin-binding mutations selectively abolished the presynaptic and postsynaptic increases in apparent synapse size (Figure 4). Blocking α-neurexin binding, even if β-neurexin binding was retained, was sufficient to abrogate the neuroligin-1 induced increase in synapse size. In addition, the dimerization mutation also abolished this effect.

Our data indicate that neuroligin-1 increases the synapse density independent of neurexin binding and dimerization, but trans-synaptically enhances the apparent synapse size in a manner dependent on α-neurexin binding and dimerization. As this conclusion is based on only a single presynaptic marker (synapsin), and on transfected neuroligin-1 as a postsynaptic marker, we sought to confirm it with additional pre- and postsynaptic markers. For this purpose, we examined an array of pre- and postsynaptic proteins in neurons transfected with either mVenus alone, wild-type neuroligin-1, or the NL1–32 neuroligin-1 mutant that abolishes α- and β-neurexin binding (Figures 5 and 6).

We found that consistent with earlier results (Chubykin *et al*, 2007), only the density of excitatory, but not inhibitory synapses was enhanced by wild-type and mutant neuroligin-1. Strikingly, all presynaptic markers examined in excitatory synapses (VGLUT1, synaptotagmin-1, synaptophysin-1, Bassoon, Munc13-1, Rab3, and synapsin) revealed a large increase in apparent synapse size (up to 100%) when wild-type neuroligin-1, but not 1–32 mutant neuroligin-1, was transfected (Figures 4–6; Supplementary Figure S7). Moreover, four of five postsynaptic markers tested (PSD-95, EGFP-actin, GluR1, and NMDA-receptor subunit 1) also displayed an increase in synapse size (30–80%; Figures 5 and

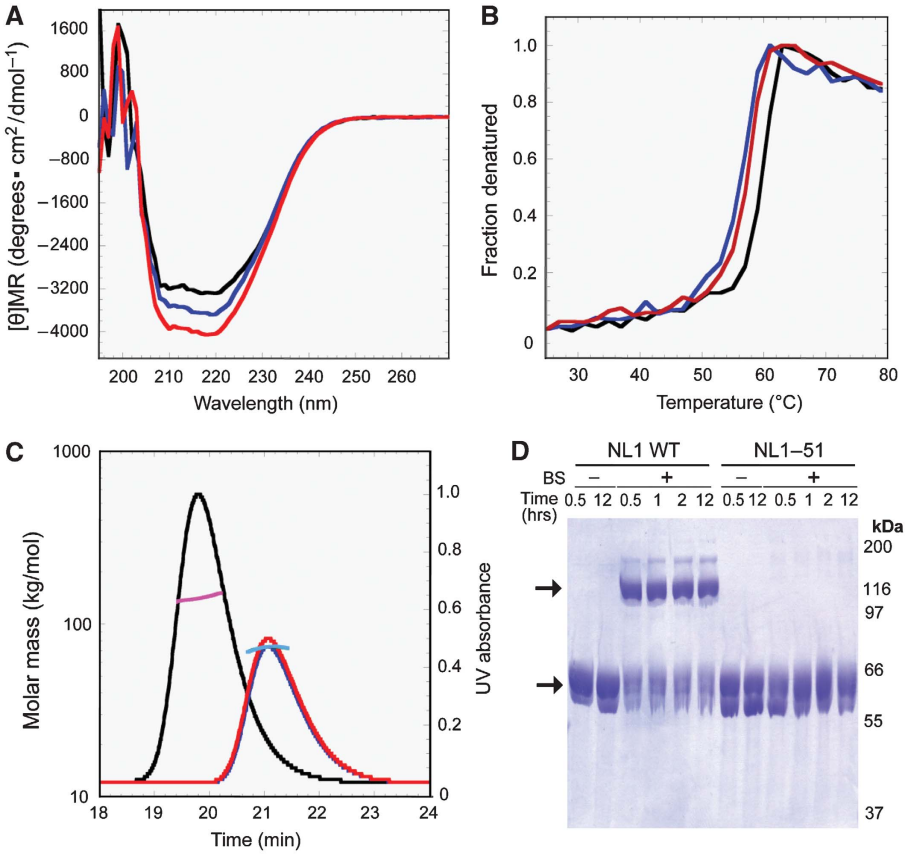

Figure 2 Characterization of neuroligin-1 dimerization mutants. (**A**) Circular dichroism spectra of wild-type (black), NL1–40 mutant (blue), or NL1–51 mutant neuroligin-1 (red), plotted as the mean residue ellipticity versus wavelength. (**B**) Temperature denaturation experiments of wild-type (black), NL1–40 mutant (blue), or NL1–51 mutant neuroligin-1 (red) measured by circular dichroism at 220 nm (T_m:wild type = ~59°C, 1–40 = 57°C; 1–51 = 58°C). The fractional denaturation was calculated by dividing the decrease in the CD signal at any time by the decrease in the CD signal that is achieved at maximum denaturation. (**C**) Combined size-exclusion chromatography and multi-angle laser light scattering of wild-type (black and magenta, respectively), NL1–40 mutant (blue and cyan, respectively), or NL1–51 mutant neuroligin-1 (red and green, respectively). Determined sizes: wild type = 135.5 kDa; NL1–40 = 66.5 kDa; NL1–51 = 67.3 kDa. (**D**) Chemical crosslinking of wild-type and 1-51 mutant neuroligin-1, treated with 0.5 mM of bis(sulfosuccinimidyl) suberate-d_0 (BS) for the indicated amounts of time. Image depicts a coomassie-stained SDS-gel; arrows point to neuroligin-1 monomers and dimers; positions of molecular weight markers are indicated on the right.

6). These effects were confirmed in triple-labelling experiments in which only synapses that are simultaneously positive for postsynaptic PSD-95 and presynaptic VGLUT1 were included in the analysis (Figure 6A and B).

Finally, to ensure that the morphologically analysed synapses with the increased synaptic-vesicle protein signal are actively being used, we used the synaptotagmin-antibody uptake assay (Figure 6C and D). In this assay, an antibody to the lumenal sequence of synaptotagmin-1 is used to follow synaptic-vesicle exo- and endocytosis (Matteoli *et al*, 1992). The data show that the density of synapses with actively recycling presynaptic vesicles is dramatically increased in postsynaptically transfected neurons expressing wild-type neuroligin-1 or the NL1–32 mutant neuroligin-1 that does not bind α- and β-neurexins (100% increase). The amount of synaptotagmin-1 antibody taken up during stimulation, however, is equally dramatically increased only in synapses contacting postsynaptic neurons expressing wild-type neuroligin-1 (again, a 100% increase), but not in synapses contacting postsynaptic neurons expressing the NL1–32 mutant neuroligin-1 (Figure 6C and D). Thus, together these data show that overexpression of neuroligin-1 in neurons induces two mechanistically distinct effects: an increase in the density of excitatory synapses on these neurons that is independent of neurexin binding and dimerization, and an increase in apparent synapse size that requires both α-neurexin binding and dimerization, and that induces a retrograde presynaptic effect.

Neurexin binding is dispensable for increases in synaptic strength induced by neuroligin-1 overexpression

We next examined the electrophysiological properties of transfected neurons expressing wild-type or mutant neuroligin-1. Both had no effect on spontaneous miniature excitatory or spontaneous miniature inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively; Figure 7A and F), a surprising result considering the increase in synapse density induced by these molecules (Figures 4–6). As described earlier (Chubykin *et al*, 2007), however, wild-type neuroligin-1 caused a selective increase in evoked excitatory, but not inhibitory synaptic transmission induced by action potentials, measured as excitatory and inhibitory postsynaptic currents, respectively (EPSCs and IPSCs, respectively; Figure 7B, D and G).

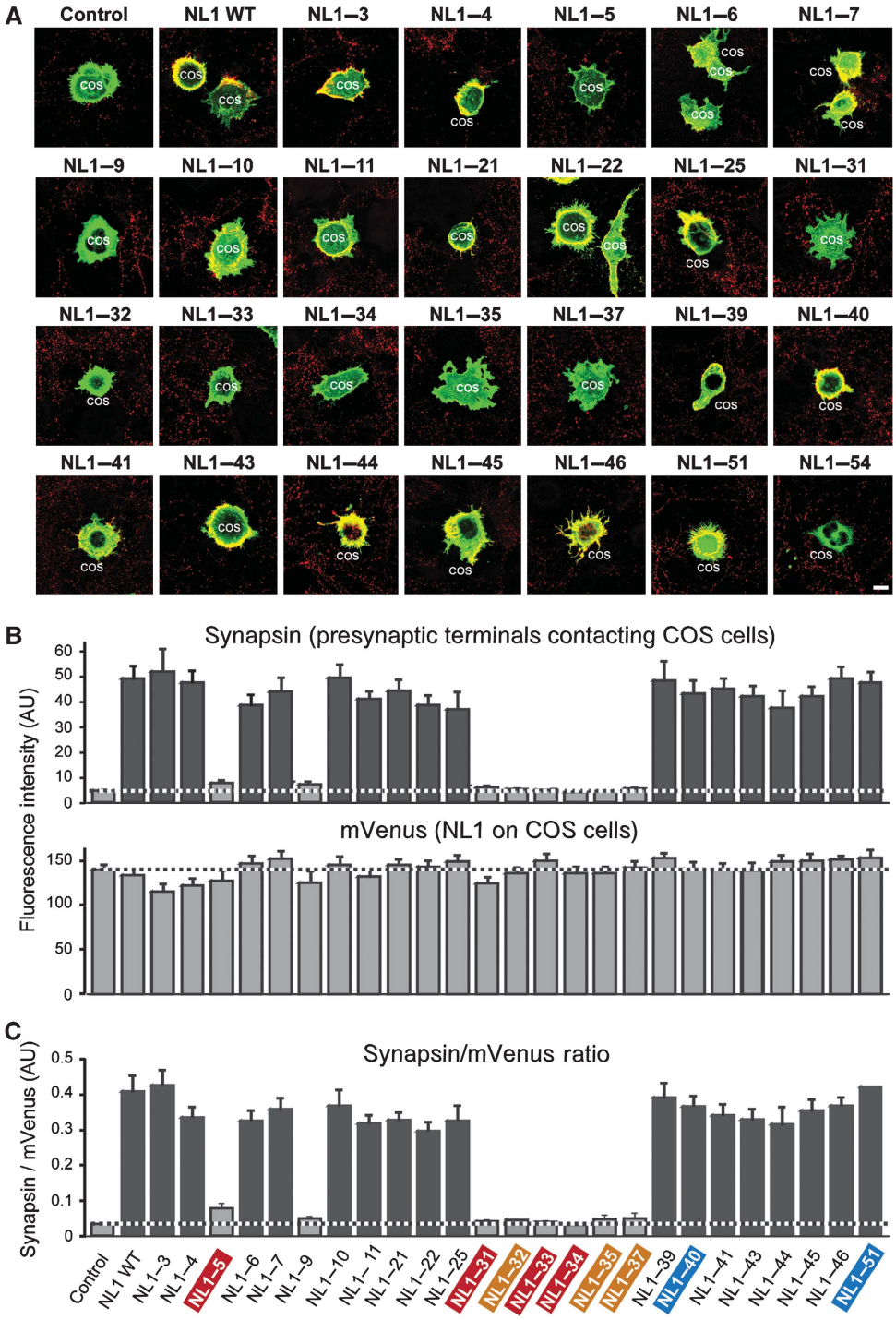

Figure 3 Synapse induction by neuroligin-1 mutants expressed in transfected COS cells. (**A**) Representative images of COS cells that were transfected with mVenus alone (control), wild-type neuroligin-1 (NL1), or the indicated point mutants of neuroligin-1 (see Table I). Transfected COS cells were co-cultured with hippocampal neurons, and examined by double immunofluorescence using antibodies to GFP to identify the transfected mVenus or the mVenus neuroligin-1-fusion proteins (green) and antibodies to synapsin to identify synapses (red). Coincident labelling of red synapses on green transfected COS cells is depicted in yellow (scale bar = 30 μm, applies to all images). (**B**) Quantitative analysis of the fluorescence intensities for synapsin over the transfected COS cells, and for mVenus in the transfected COS-7 cells co-cultured with neurons (AU = arbitrary units; dashed lines = mVenus signal as the baseline). Note that neuroligin-1 mutants that lack neurexin binding failed to induce synapse formation. (**C**) Normalized synapse density on transfected COS cells co-cultured with neurons, expressed as the ratio of synapsin staining to mVenus fluorescence. In (B–C), data shown are means ± s.e.m. ($n = 3$–5 independent experiments). Names for neurexin-binding-deficient mutants are coloured red (selectively α-neurexin-binding-deficient) or orange (α- and β-neurexin-binding-deficient), and for dimerization mutants blue. Grey bars are not statistically significantly different from the mVenus-only control; black bars are statistically significantly different at $P < 0.01$.

Moreover, wild-type neuroligin-1 increased the NMDA/AMPA ratio (~40% enhancement; Figure 7C), consistent with Chubykin *et al* (2007). Again, the neurexin binding or dimerization mutations had no effect on the increase in synaptic strength or NMDA/AMPA ratio induced by overexpressed neuroligin-1 (Figure 7).

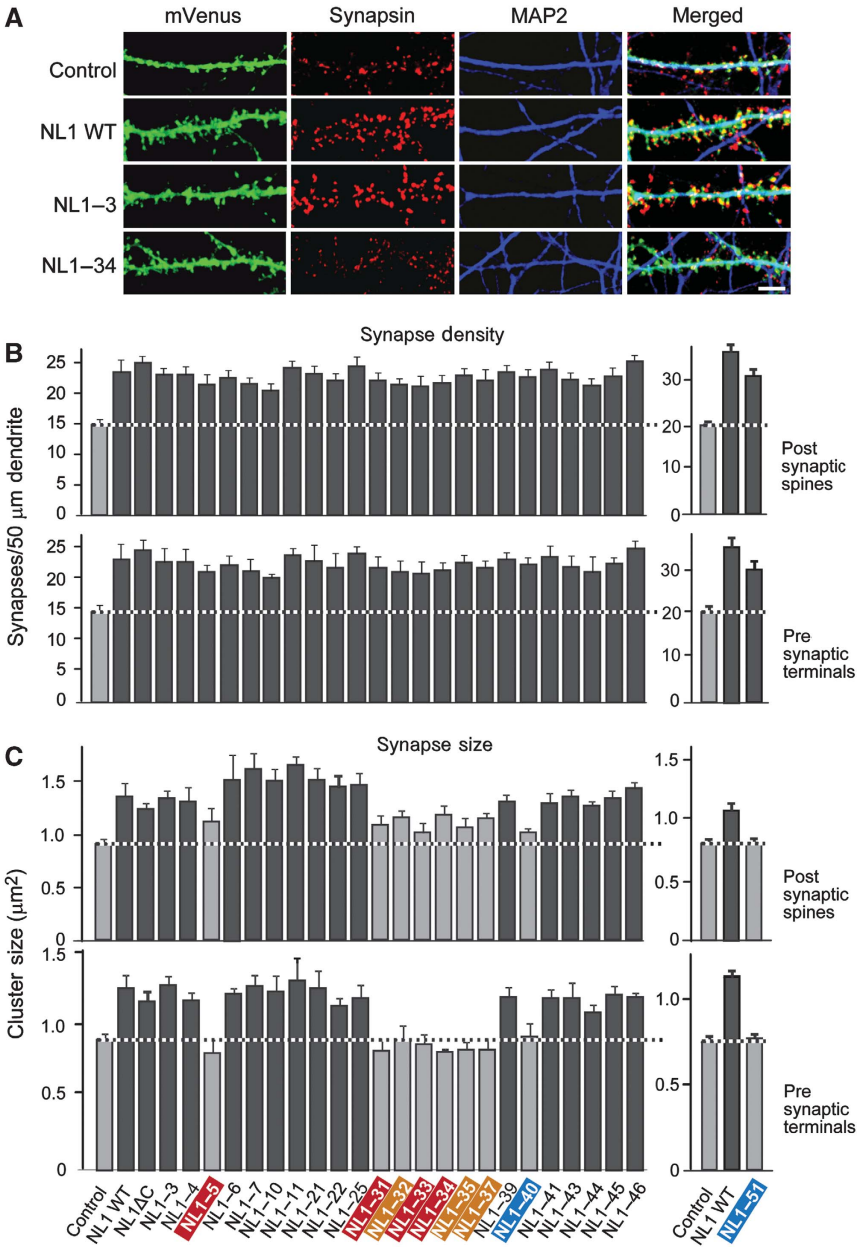

Figure 4 Synapse induction and modification by neuroligin-1 mutants expressed in transfected neurons. (**A**) Representative images of neurons transfected with mVenus only (Control), or mVenus-fusion proteins of wild-type neuroligin-1 (NL1 WT) and the NL1–3 or NL1–34 mutants of neuroligin-1. Transfected neurons were visualized by triple immunofluorescence labelling for mVenus contained in the transfected neuroligins (green; left), the presynaptic marker synapsin (red; left centre), and the dendritic marker MAP2 (blue; right centre). Merged images are shown on the right (white = coincident signal; scale bar = 5 µm, applies to all images). For additional representative images, see Supplementary Figure S6. (**B**, **C**) Quantitations of synapse density (B) and apparent synapse size (C) on transfected neurons expressing mVenus or the neuroligin-1 constructs listed at the bottom. Data for NL1–51 obtained in separate experiments are shown separately on the right. Synapse density was measured either as the density of light-microscopically identifiable spines (B, top) or of synapsin-positive presynaptic terminals that contact the transfected neuron (B, bottom). The apparent synapse size (C) was measured as the relative fluorescence signal intensity for either the postsynaptic neuroligin-1-fusion protein or the presynaptic synapsin staining. Data shown are means ± s.e.m. ($n = 3$–9 independent experiments; colour scheme is the same as in Figure 3). Grey bars are not statistically significantly different from the mVenus-only control; black bars are statistically significantly different at $P < 0.05$.

To investigate whether the increased evoked EPSC after neuroligin-1 overexpression is because of an increase in release probability or in the readily releasable pool (RRP), we measured the paired-pulse ratio of closely spaced EPSCs (as an indirect measure of release probability), and the size of the EPSC induced by application of hypertonic sucrose (which triggers synaptic-vesicle exocytosis in a Ca^{2+}-independent manner and is thought to allow measurements of the entire RRP of the synapses on a neuron; Rosenmund and Stevens, 1996). We found that neuroligin-1 overexpression—wild-type or mutant—enhanced the RRP (Figure 7E), but did not change the paired-pulse ratio (Supplementary Figure S8). The effects of wild-type and NL1–32 mutant neuroligin-1 on the RRP and on action-potential-evoked EPSCs were similar. These results suggest that neuroligin-1 increases the overall synaptic capacity on the neuron, despite its lack of an effect

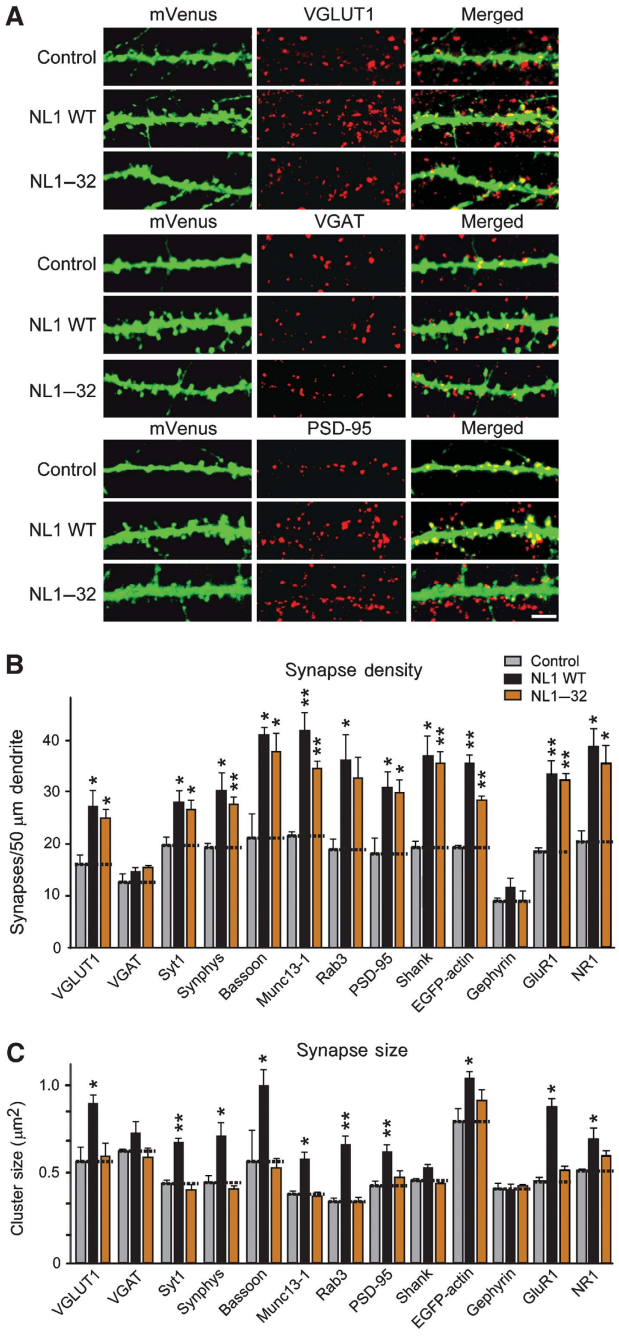

Figure 5 Analysis of synapse enhancement by neuroligin-1 using multiple markers. (**A**) Representative images of neurons transfected with mVenus alone (Control), wild-type neuroligin-1 (NL1 WT), or 1–32 mutant neuroligin-1 (NL1–32). Neurons were visualized by double immunofluorescence labelling for mVenus (green) and various pre- and postsynaptic markers as indicated (red; for representative images with additional markers, see Supplementary Figure S7). (**B, C**) Quantitation of synapse density (B) and size (C) in neurons expressing mVenus alone (grey), wild-type neuroligin-1 (black), or 1–32 mutant neuroligin-1 (orange), using the indicated pre- or postsynaptic marker proteins. Data shown are means ± s.e.m. ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ in pairwise comparisons between control and experimental groups).

on the spontaneous miniature release rate, by a mechanism that is independent of neurexin binding. These results also suggest that the observed neurexin-dependent increase in apparent synapse size does not alter the global physiological properties of the synapses on a neuron.

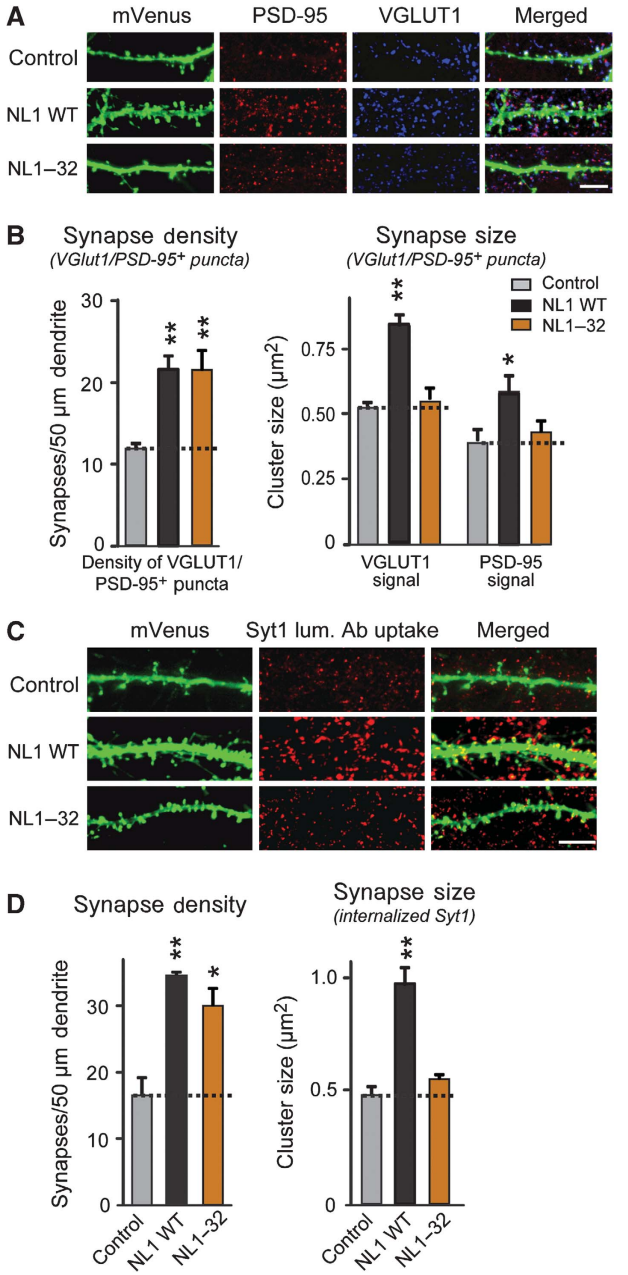

Figure 6 Neurexin binding by neuroligin-1 is required for potentiation of the size of functional presynaptic terminals. (**A**) Representative images of neurons transfected with mVenus alone (Control), wild-type neuroligin-1 (NL1 WT), and NL1–32 mutant neuroligin-1. Neurons were triply stained with mVenus (green), PSD-95 (red), and VGLUT1 (blue). Scale bar = 5 µm, applies to all images. (**B**) Quantitation of the density and size of synapses on transfected neurons in multiple experiments as described in (A); only synapses that are positive for both VGLUT1 and PSD-95 were included. (**C**) Representative images of neurons transfected with mVenus alone (Control), wild-type neuroligin-1 (NL1 WT), or NL1–32 mutant neuroligin-1 (NL1–32). Neurons were incubated in the presence of antibodies to the lumenal sequence of synaptotagmin-1 (Perin *et al*, 1991) for 10 min in high K^{+} medium (57 mM; Matteoli *et al*, 1992). Afterwards, neurons were fixed, and analysed by double immunofluorescence for mVenus (green, to label the transfected proteins) and the synaptotagmin-1 antibody (red, to label synaptic vescicles undergoing exo- and endocytosis). Scale bar = 5 µm, applies to all images. (**D**) Quantitation of multiple experiments performed as described in (C). Data shown in (B) and (D) are means ± s.e.m. ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ in pairwise comparisons between the control and experimental groups).

Discussion

Multiple recent studies showed that neuroligin-1 is a post-synaptic cell-adhesion molecule that has an important function in shaping central synapses in brain, and that neuroligin-1 acts by binding to neurexins (e.g. Song *et al*, 1999; Dean *et al*, 2003; Varoqueaux *et al*, 2006; Chubykin *et al*, 2007). Moreover, neuroligin-1 forms constitutive homodimers (Comoletti *et al*, 2003), leading to the postulate that its signal is transduced through dimerization of neurexins (Dean *et al*, 2003). The goal of this study was to directly test these hypotheses. Multiple effects of neuroligin-1 on synapses were earlier characterized, prompting us to ask which of these effects, if any, require binding of neurexins. Moreover, if these effects were mediated by neurexin binding, we asked whether they are mediated by neuroligin dimerization. To address these questions, we used a well-established approach developed in earlier studies of neuroligin-1 function: the ability of neuroligin-1, when displayed on a transfected non-neuronal cell, to induce synapse formation from co-cultured neurons (Scheiffele *et al*, 2000; Chubykin *et al*, 2005), and the ability of neuroligin-1, when transfected into a neuron, to increase the density, apparent size, and strength of synapses on these neurons (Chih *et al*, 2004; Boucard *et al*, 2005; Levinson *et al*, 2005; Chubykin *et al*, 2007).

Our results unequivocally establish that neurexin binding by neuroligin-1 is important because it is essential for its ability to induce synapses on a non-neuronal cell (Figure 3). A block of α-neurexin binding—with continued β-neurexin binding—already abolishes this activity of neuroligin-1. However, we found that neurexin binding is not required for neuroligin-1's ability to enhance the density of synapses in a transfected neuron, even when both α- and β-neurexin binding are blocked (Figures 4–6). These two findings lead to four conclusions, of which only the first conclusion was expected. First, as postulated before, neuroligin-1 forms a trans-synaptic cell-adhesion complex with presynaptic α-neurexins in the artificial synapse-formation assay. Second, the activity of neuroligin-1 that is revealed in the artificial synapse-formation assay is different from its activity in potentiating the synapse density on a neuron overexpressing it, which does not require neurexin binding; thus, these two activities represent distinct synaptic processes. Third, neuroligin-1 must interact with additional presynaptic ligands, directly or indirectly, to account for its various

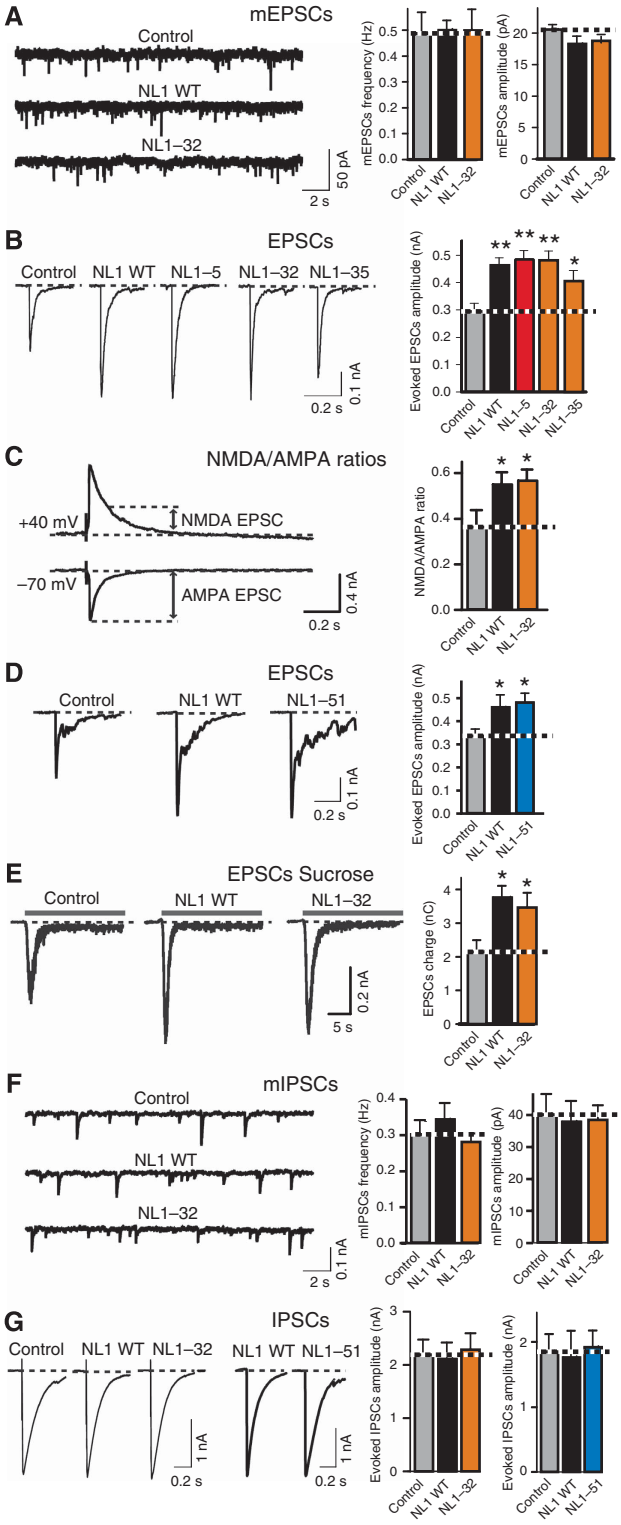

Figure 7 Electrophysiological effects of neuroligin-1 mutants in transfected neurons. Neurons were transfected with mVenus alone (Control), with wild-type neuroligin-1 (NL1 WT), various neurexin-binding mutants of neuroligin-1 (NL1–5, –32, and –35), or the NL1–51 dimerization-defective mutant of neuroligin-1. (**A**) Representative traces (left) and bar diagrams of the frequency (centre) and amplitude (right) of miniature EPSCs. (**B**) Representative traces (left) and diagram bars of the amplitude (right) of action-potential-evoked AMPAR-dependent EPSCs. (**C**) Representative traces of NMDA- and AMPA-receptor-dependent EPSCs (left), and bar diagrams of the measured NMDA/AMPA-receptor EPSC ratio (right). (**D**) Representative traces (left) and diagram bars of the amplitude (right) of action-potential-evoked AMPAR-dependent EPSCs. (**E**) Representative traces (left) and mean charge transfer (right, integrated over 30 s) of EPSCs elicited by hypertonic sucrose (0.5 M sucrose for 30 s). (**F**) Representative traces (left) and diagram bars of the frequency (centre) and amplitude (right) of miniature IPSCs in neurons expressing mVenus, wild-type neuroligin-1 (NL1 WT), or NL1–32 neurexin-binding mutant of neuroligin-1 (NL1–32). (**G**) Representative traces (left) and diagram bars of the amplitude (right) of action-potential-evoked IPSCs. Recordings were from the transfected neurons identified by mVenus fluorescence. The scale bars apply to all traces in a set. Data shown are means ± s.e.m. Asterisks above the bar diagrams indicate statistically significant differences in pairwise comparisons between the control and experimental groups ($n = 3$–8 independent experiments; $^*P < 0.05$, $^{**}P < 0.01$). Dashed and dotted lines refer to the values of mVenus as a negative control.

activities. This conclusion, although unexpected, is consistent with the earlier finding that both neuroligin-1 and -2 bind to neurexins, but that the former selectively enhances excitatory, and the latter inhibitory, synaptic function (Chubykin *et al*, 2007). Fourth, finally, for the artificial synapse-formation assay and the effect of neuroligin-1 on apparent synapse size, β-neurexin binding is not sufficient, but α-neurexin binding is required.

We also found that although neurexin binding by neuroligin-1 is not essential for increasing the density of synapses on a transfected neuron, it is required for increasing the apparent size of these synapses (Figures 4–6). We measured this increase and its dependence on neurexin binding with a series of pre- and postsynaptic markers to confirm that this observation was not an artefact of one particular marker (Figures 5 and 6). Overall, both presynaptic-vesicle markers and active zone markers revealed a dramatic increase in synapse size, as measured by immunofluorescence, showing that this effect was consistently observed presynaptically. Although the same effect was also detected postsynaptically with four out of five markers tested, it was not as large (Figures 5 and 6). The observation of large presynaptic apparent size increases induced by binding of postsynaptically overexpressed neuroligin-1 suggests, consistent with the artificial synapse-formation assay discussed above, that postsynaptic neuroligin-1 triggers a retrograde presynaptic signal by binding to α-neurexins. It should be noted, however, that the interpretation of the size effect is not straightforward. We are measuring the strength of the immunofluorescence signal; although the software of the confocal microscope interprets this as a measure of synapse size, it could also consist of an increase in the density of synaptic antigens in the synapse without a change in synapse size. To differentiate between these two possibilities, quantitative immunoelectron microscopy would be required, which is difficult because the few transfected neurons cannot be readily identified. Independent of which possibility is true, our data confirm that postsynaptic neuroligin-1 signals trans-synaptically by activating presynaptic neurexins through dimerization. In neurons, this signal is not involved in synapse formation as such, consistent with knockout results (Missler *et al*, 2003), but regulates the presynaptic properties of the synapses on a neuron expressing neuroligin-1.

Our results are at odds with earlier data suggesting that dimerization by neuroligin-1 is important for the ability of neuroligin-1 to exit the endoplasmic reticulum and to induce increases in synapse abundance in cultured neurons and in the artificial synapse-formation assay (Comoletti *et al*, 2003; Dean *et al*, 2003). However, in earlier studies, we and others did not use neuroligin-1 mutants whose correct folding was assured, and whose monomeric nature was confirmed biophysically. Key mutants used in this study, such as the dimerization mutant NL1–51 and the neurexin binding impaired mutant NL1–32, were analysed by multiple structural and spectroscopic methods to show that they are not partly destabilized or misfolded. With the recent structural information (Arac *et al*, 2007; Fabrichny *et al*, 2007; Chen *et al*, 2008) that was not available for the earlier studies, it seems likely that the earlier mutations caused structural perturbations, which may have impaired neuroligin-1 transport to the cell surface and its function beyond the intended abolition of dimerization.

In summary, our study confirms the exquisite specificity of neuroligin-1 in boosting excitatory synaptic inputs on a neuron with little effect on inhibitory synapse numbers and synaptic strength, but shows that the actions of neuroligin-1 must be mediated by multiple independent mechanisms that validate synapses in different ways, presumably by stabilizing transient synapses (which would account for the apparent synapse induction activity of neuroligin-1), and by altering the properties of synapses (which would account for the change in apparent synapse size and NMDA/AMPA ratio induced by neuroligin-1). Elucidating the protein–protein interactions involved in all of these mechanisms forms a challenge that simultaneously provides an opportunity to identify additional synaptic cell-adhesion events.

Materials and methods

For detailed experimental procedures and lists of reagents, please see the Supplementary data.

Cell-surface-binding assays

This was performed with HEK293T cells (Boucard *et al*, 2005), using purified Ig-fusion proteins that were bound to transfected HEK293T cells expressing wild-type or mutant neuroligin-1 or mVenus only. Bound Ig neurexins were detected using anti-rabbit Alexa fluor 633 antibodies (Molecular Probes), imaged by confocal microscopy (TCS2, Leica), and analysed by comparison of Ig-neurexin binding to the neuroligin-1 expression levels measured through the mVenus fluorescence of the neuroligin-1 mVenus-fusion proteins.

Antibody labelling and surface biotinylation assays

This was performed with transfected HEK293T cells (Chubykin *et al*, 2005), using monoclonal antibodies 4C12 (Song *et al*, 1999) or 2 mg/ml of Sulfo-NHS-LC-biotin (Pierce).

Artificial synapse-formation assays

This was performed with COS-7 cells (Supplementary Table 1; Chubykin *et al*, 2005).

Neuronal cultures, transfections, immunocytochemistry, image acquisition, and analyses

Primary rat hippocampal neurons were cultured from E18 embryos (Maximov *et al*, 2007), transfected at DIV10, and immunostained at DIV14 after fixation with 4% paraformaldehyde/4% sucrose and permeabilization with 0.2% Triton X-100. For measuring synaptic-vesicle exo- and endocytosis, transfected neurons were incubated with synaptotagmin-1 lumenal antibodies (CL604.4, 1:10; Matteoli *et al*, 1992). Images of randomly chosen transfected neurons were acquired with a confocal microscope (LSM510, Zeiss or TCS2, Leica) at constant settings. Z-stacked images obtained from confocal microscope were converted to maximal projection and analysed using MetaMorph Software (Molecular Devices) with area size and density of spines and presynaptic terminals per 50 μm of dendrite. To quantify the synaptic puncta size, we thresholded all the images equally and measured the average pixel intensities along the dendritic segments in the transfected neurons by manually tracing each puncta with >two-fold background signal. All numerical values for the morphometric results are listed in Supplementary Table 1.

Electrophysiological recordings

This was performed in transfected rat neurons cultured as mixed cells at high density, using whole-cell recordings (Maximov *et al*, 2007). Spontaneous miniature and evoked EPSCs and IPSCs were pharmacologically isolated; tetrodotoxin (1 μM) was added to the bath to block evoked synaptic responses for mEPSC or mIPSC recordings. The RRP was measured by gravity perfusion of the recording chamber with hypertonic sucrose (0.5 M) at 2 ml/min. Recordings with series resistances of >20 MΩ were discarded. Numerical values for all electrophysiology results are listed in Supplementary Table 2.

Biophysical experiments

This was performed as described (Arac *et al*, 2007 and Supplementary data).

Statistics

Statistical significance was determined by Student's *t*-test. All experiments were repeated independently at least three times; all values are expressed as means ± s.e.m. The number of independent transfection experiments was used as the basis ('*n*') for the statistical analyses of Figures 3–6.

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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