Dystroglycan Binding to α–Neurexin Competes with Neurexophilin-1 and Neuroligin in the Brain*

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Background: Extracellular matrix dystroglycan has essential functions at the neuromuscular junction and at inhibitory synapses in the brain.

Results: Brain dystroglycan competes with neurexophilin-1 and neuroligin for binding to presynaptic α-neurexins.

Conclusion: Competition between α-neurexin ligands in combination with alternative splicing determines formation of important trans-synaptic complexes.

Significance: This is the first analysis of binding interference in α-neurexin multiplexes.

SUMMARY

α-Neurexins (α-Nrxn) are mostly presynaptic cell surface molecules essential for neurotransmission that were linked to neurodevelopmental disorders as autism or schizophrenia. Several interaction partners of α-Nrxn were identified that depend on alternative splicing, including neuroligins (Nlgn) and dystroglycan (α-DAG). The trans-synaptic complex with Nlgn1 was extensively characterized and shown to partially mediate α-Nrxn function. However, the interactions of α-Nrxn with α-DAG, neurexophilins (Nxph1) and Nlgn2, ligands that occur specifically at inhibitory synapses, are incompletely understood. Using site-directed mutagenesis, we demonstrate the exact binding epitopes of α-DAG and Nxph1 on Nrxn1α, and show that their binding is mutually exclusive. Identification of an unusual cysteine bridge pattern and complex type glycans in Nxph1 ensure binding to the second laminin/neurexin/sex-hormone-binding (LNS2) domain of Nrxn1α, but this association does not interfere with Nlgn binding at LNS6. α-DAG, in contrast, interacts with both LNS2 and LNS6 domains without inserts in splice sites SS#2 or SS#4 mostly via LARGE-dependent glycans attached to the mucin region. Unexpectedly, binding of α-DAG at LNS2 prevents interaction of Nlgn at LNS6 with or without splice insert in SS#4, presumably by sterically hindering each other in the u-form conformation of α-Nrxn. Thus, expression of α-DAG and Nxph1 together with alternative splicing in Nrxn1α may prevent or facilitate formation of distinct trans-synaptic Nrxn/Nlgn complexes, revealing an unanticipated way to contribute to the identity of synaptic subpopulations.

Neurexins (Nrxn) are transmembrane proteins that localize primarily to presynaptic terminals (1). Nrxn are essential for Ca²⁺-dependent transmission at excitatory and inhibitory synapses in the central and peripheral nervous system (2-6), and play additional roles in synapse formation and differentiation (7-12). All three vertebrate Nrxn genes (Nrxn1-3) encode two major isoforms: extracellularly longer α-Nrxn and shorter β-Nrxn which are transcribed from
independent promoters but share most exons (13). α-Nrxn proteins contain six LNS (laminin-neurexin-sex hormone binding globulin) domains with three interspersed EGF (epidermal growth factor-like) domains. β-Nrxn have a unique N-terminal stretch of 37 histidine-rich residues but are identical to α-Nrxn starting from LNS6 and ending in a cytosolic domain with PDZ binding motif that is required for trafficking (14). LNS domains are structurally characterized by a β-sheet sandwich (15-18), a core fold similar to the concanavalin A family (19). LNS domains are thought to behave like glycan-binding lectins (18), and LNS domains of Nrxn, laminin and agrin have similar Ca\(^{2+}\)-coordination sites (20). Nrxn LNS2 and LNS6 are distinguished by hydrophobic residues near the Ca\(^{2+}\)-binding site, providing specific interfaces for binding partners (20,21). Moreover, Nrxn contain up to six alternative splice sites in α -Nrxn (SS#1-6) and two in β -Nrxn (SS#4,5) (13,22). Nrxn alternative splicing is physiologically relevant because it controls aspects of their function (23,24) and binding to postsynaptic Nlgn (25,26).

Splice insert-dependent formation of the Nrxn/Nlgn complex is intricate because Nlgn also has two splice sites. Nlgn1 contains SS_A and SS_B (26), Nlgn2 and Nlgn3 carry only SS_A (27), and Nlgn4 is not alternatively spliced (28). Co-crystal data exist for the interface between Nrxn LNS6/βLNS lacking insert in SS#4 (-SS#4) with Nlgn1 and Nlgn4 (15-17). Nlgn3 is predicted to form similar complexes (15-17), whereas Nlgn2 differs structurally with a G500Q change and may use another epitope (17,29). Affinity purification of Nlgn with the extracellular domain of β-Nrxn originally suggested that only β -Nrxn(-SS#4) binds Nlgn1 (26). This apparent restriction fostered the idea of a splice code in Nrxn/Nlgn complexes (11,25,26,30,31). Subsequently, it was shown that all Nrxn, including α-Nrxn(+SS#4), are able to bind to Nlgn1(-B) or Nlgn2 due to displacement of the insert (20,25,32,33).

In addition to Nlgn, other extracellular partners of Nrxn were identified, most notably neuroxophilin (Nxph) (34-36), dystroglycan (DAG) (37), leucine-rich repeat proteins (LRRTM2) (38,39) and cerebellin (40,41). Remarkably, all of these ligands bind at only two Nrxn domains, LNS2 and LNS6/βLNS. While Nxph binds LNS2 independently of alternative splicing (34), DAG and LRRTM require splice insert-free LNS domains (37,42) and cerebellin binds directly to the insert in SS#4 (40,41).

Unlike Nrxn variants that are expressed in most excitatory and inhibitory neurons (43), the α-Nrxn-specific ligand Nxph1 is restricted to inhibitory interneurons (36,44), similar to α-DAG which also prefers subsets of inhibitory synapses where it may co-localize with Nlgn2 (45-48). Nxph comprise a family of glycoproteins (Nxph1-4) that exhibit characteristics of secreted, preproprotein-derived molecules (35,36) but the structural determinants of their interaction with LNS2 (34) are still unclear. DAG in turn is produced from an evolutionarily conserved single gene (49) and proteolytically cleaved into extracellular α-DAG and transmembrane β-DAG that remain non-covalently attached (50). While specificity of α-DAG binding to matrix proteins such as laminin comes from glycosylation of distinct residues in the mucin-rich regions (51,52), the glycan moiety required for association of Nrxn (37,53) is undetermined.

Here, we present the distinct interaction sites of Nxph1 and α-DAG at the LNS2, and study their crosstalk with ligands of the LNS6 domain of α-Nrxn. Surprisingly, we observed that binding of α-DAG and Nxph1 is mutually exclusive, and that association of α-DAG at LNS2 prevents formation of the trans-synaptic complex with Nlgn at LNS6. These are important results because impairments in α-Nrxn/Nlgn complexes is linked to neurodevelopmental disorders (54-56), and there is symptomatic overlap with cognitive defects observed in DAG-associated muscular dystrophy syndromes (53,57,58).

**EXPERIMENTAL PROCEDURES**

*Molecular cloning and expression constructs—*

Previously described plasmids used include: full-length rat Nrxn1α, pCMVL2; Fc-tagged extracellular domains of Nrxn1α, pCMVlgN1α-1; Fc-tagged Nrxn1β, pCMVlgN1β-1 & pCMVlgN1β-3 (59); Fc-control (IgG) vector, pCMVlgNrxnSP; pCMVlgLNS6-1 without and pCMVlgLNS6-3 with insert in SS#4; mutations in LNS6-1 GT1201VA, LIN1280/2/4SSD, T1281A, D1183A; cassette pCMVlgLNS5-EGF3-LNS6; extracellular domains of rat Nlgn1 without insert B, pCMVNL1-B (20); full-length rat Nxph1, pCMVD2 (36); rat Nlgn2, pCMVNL2-1 (27); full-
length rat DAG, pCMVDAG (60); full-length human LARGE, pCMV6-XL4 LARGE (OriGene Technologies).

Novel plasmids generated for this study include: (i) Nrxn1α-constructs: single LNS domains (LNS1 to LNS6) and cassettes (LNS1-EGF1-LNS2, LNS3-EGF2-LNS4) were amplified from pCMV2L, and cloned into pCMVlgNrxnSP to add a C-terminal Fc-tag. Site-directed mutagenesis on template Fc-LNS2 was done by Quikchange (Agilent Technologies, Santa Clara, USA) to introduce mutations: C293S, SS304-305AA, Q316V, S327A, D329A, G347A, S348A, V358D, E356A, N359A, A366W, T395D, I401D, T403D, TTT403-405PPP, T404P, QED409-411AAA, Y412A, Y412F, YMG412/14/16SSD, D418A, D418R, D419A, D419R, F420D, G421A, G421D, Y422A, Y422F, YMG422/14/16SSD, TTT432-434PPP, T433P, QED437-439AAA, E356A, N359A, A366W, T395D, I401D, T403D, TTT403-405PPP, T404P, QED409-411AAA, Y412A, Y412F, YMG412/14/16SSD, D418A, D418R, D419A, D419R, F420D, G421A, G421D, Y422A, Y422F, YMG422/14/16SSD, TTT432-434PPP, T433P, QED437-439AAA, E356A, N359A, A366W, T395D, I401D, T403D, TTT403-405PPP, T404P, QED409-411AAA, Y412A, Y412F, YMG412/14/16SSD, YMG412/14/16L1N, T413A, M414A, G416A, G416N, D418A, DD418-419AA, F420D, Δ542-436P, L462R, and I472P. Mutation I1282R was introduced in Fc-LNS2 and Fc-LNS6 (LNS6-HA), HA sequence followed by a stop codon was introduced in Fc-LNS2 and Fc-LNS6. Soluble, non-tagged extracellular domains of Nrxn1α (aa 31-LEF...EST-1346) (+SS#4 & -SS#4) were created by inserting a stop codon in pCMVL2, and cloned into pCMVIgNrxnSP to add a C-terminal Fc-tag. Site-directed mutagenesis on was done to deglycosylate αDAG at N139, T315 or T317, and to delete either half or the complete mucin region (Fc-αDAG-N139D, Fc-αDAGmuc2 (Δmuc1), Fc-αDAGmuc1 (Δmuc2), Fc-αDAGmuc, Fc-αDAG-T315A/T317A). A soluble αDAG with C-terminal HA-tag was created by inserting an HA-stop sequence (αDAG-HA).

All enzymes for restriction sites, dephosphorylation, ligation and appropriate buffers were purchased from New England Biolabs (Ipswich, MA, USA). Custom-made Primers were made by Sigma (München). PCR was carried out with iProof™ high fidelity PCR (Bio-Rad, München), and DNA fragments isolated using phenol-chloroform extraction or QiaEx (Qiagen, Hilden). All resulting intermediaries and final constructs were confirmed by DNA sequencing (GATC, Konstanz).

Biochemical procedures—For expression of Fc-tagged recombinant proteins, constructs were transfected into HEK293, tsA-201 or N2a cells using calcium phosphate. Briefly, 28 µL of 8.4 - 17 µg DNA in TE (10mM Tris pH 8, 1mM EDTA) was pre-mixed with 672 µL 150mM CaCl2 and 700 µL phosphate buffer (274mM NaCl, 12mM glucose, 10mM KCl, 1.4mM Na2HPO4, 40mM HEPES pH7.04), incubated at RT for 20min and added to 1.4*10^6 HEK cells in growth medium (DMEM, 10%FCS, 5% penicillin/streptomycin). Medium was changed to FCS-free medium after 24 hrs, and recombinant proteins harvested 72 hrs after transfection. Full-length Nrxn1α, Nlgn1, Nlgn2, and αDAG-HA were produced in COS7 cells using DEAE-dextran transfection. Briefly, 0.5*10^6 cells in 10 cm dishes were washed twice with pre-warmed (37°C) 1x TBS, transfected with 3.3 ml of 1.65ml 2x TBS, 1.25ml H2O, 66 µl DNA (0.1µg/µl) and 330 µl DEAE-dextran (5mg/ml), and incubated for 30min at 37°C, 5% CO2. Medium was changed (DMEM, 10% FCS, 1% PS), 100µM Chloroquine added, and cells were incubated for 3hrs at 37°C, 5% CO2. Medium was changed and cells incubated for 2 days before harvest. To produce deglycosylated Nxes, tunicamycin (2 µg/L) was added 24 hrs after transfection to FCS-free medium.

For co-sedimentation assays, secreted Fc-tagged proteins were bound to Protein A conjugated sepharose beads overnight, washed three times, and either analyzed directly or used in
pulldown experiments, essentially as described (20). In short, mouse brains were disrupted with a Polytron followed by Dounce homogenization in buffer H (100mM NaCl, 5 mM CaCl₂, 50mM Tris pH 7.5). Triton-X 100 was added to a final concentration of 1% (wt/vol) for 3 hrs at 4°C, followed by centrifugation at 220.000 x g for 30 min. COS-7 or N2a cell lysates were obtained from scraped cells with 1% Triton-X 100 in buffer H for 30 min at 4°C and centrifugation (15.000 x g, 1 min). Aliquots of lysate were added to purified Fc-fusion proteins in buffer H containing 0.1% Triton X-100 for binding at 4°C overnight. After washing, bound proteins were analyzed by SDS/PAGE, Coomassie staining and/or immunoblotting (Bio-Rad).

To obtain a pure Fc-Nxph1/LNS2-HA complex for mass spectrometry, we eluted free and LNS2-HA-bound Fc-Nxph1 from protein A beads with glycine buffer (50mM glycine pH 1.8) for 30min, neutralized the eluate to pH 7 with 1M Tris/HCl pH 8, separated the protein mixture on a superdex 200 gel filtration column XK 16 and collected the fraction containing only Fc-Nxph1/LNS2-HA complex using an Äkta™ prime system (GE Healthcare). The protein solution was concentrated to 500 µl by Amicon ultracel filters (3k), dialyzed against 10mM ammonium bicarbonate in Slide-A-Lyzer™ chambers (Thermo Scientific) prior to mass spectrometry analysis.

Surface plasmon resonance (SPR) analysis—Fc-Nxph1/LNS2-HA eluted from protein A beads as described above was bound covalently on a CMD 200m chip using 5mM Na-Acetate (pH 5) and EDC*NHS (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid, N-Hydroxysuccinimid) following the manufacturer’s protocol (Reichert Technologies, New York, USA). Binding occurred randomly in a flow of 50µl/min, and stochastically either Nxph1, LNS2 or both proteins were irreversibly immobilized on the chip. 50mM Tris pH 7.4 served as running buffer, and several solutions were tested to release either Nxph or LNS2 from their complexes. Complexes that were covalently linked to the chip via both proteins remained inert to elution buffers used, leading to a systematic underestimation of the eluted fraction under all conditions which does not bias our relative comparison between WT and point-mutated complexes. Measurements were done with a two-channel Reichert SR7000DC SPR System (Reichert Technologies).

Mass spectrometry—Nxph1 samples were digested in ammonium bicarbonate buffer (10mM) overnight with trypsin, chymotrypsin or a 1:1 mixture of trypsin and chymotrypsin at 37°C or with thermolysin at 65°C. Digest mixtures were dried, redissolved in water and dried again. For separation of N-glycopeptides, ZIC-HILIC ProteaTips were used as described previously (61). Products were analyzed by nanoESI Q-ToF MS using quadrupole time-of-flight (Q-Tof) mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray source in positive ion mode. Spectra were acquired at source temperature of 80°C, a desolvation gas (N2) flow rate of 75 l/h, a capillary voltage of 1.1kV, and a cone voltage of 30-40V. For low energy CID experiments, the (glyco)peptide precursor ions were selected in the quadrupole analyzer and fragmented in the collision cell using a collision gas (Ar) pressure of 3.0 × 10⁻³Pa and collision energies of 20–40eV (Elab). The (glyco)peptide structures were deduced from the resulting fragment ion spectra (table 1).

Immunoelectron microscopy—Neocortical brain tissue from wild-type mice was embedded in Lowicryl HM20 (Polysciences, Eppelheim, Germany) using freeze substitution in methanol. Anesthetized mice were transcardially perfused with 0.1% glutaraldehyde (Roth, Karlsruhe, Germany) and 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M PB at 37°C, and postfixed for 2 hrs. 300 µm thick vibratome slices were infiltrated for cryoprotection with 5% sucrose in 0.1 M PB followed by 10%, 20% and 30% glycerol in 0.1 M PB for 2 hrs each. Blocks were cut and plunged into 4% uranylacetate in 99.5% methanol precooled to -90°C in a Leica AFS2 for 12 hrs and additional 24 hrs at -45°C (slope 5°C/h). After three washing steps in methanol (30 min at -45°C) infiltration followed for 2 hrs with 50% Lowicryl in methanol, 67% Lowicryl in methanol and pure Lowicryl for additional 16 hrs. Polymerisation with UV-light proceeded for 24 hrs at -45°C and additional 24 hrs at 0°C (slope 4°C/h), and finally 24 hrs at RT (slope 0.9°C/h). Postembedding immunogold labeling on Lowicryl sections started by blocking on 2% HSA/0.05 M TBS droplets, followed by incubation with primary antibodies against Nxph1.
(1:50), Nrxn (1:50), Nlgn2 (1:50), Nlgn1 (1:50) and pan-synapsin (1:50) overnight at 4°C, and 10 nm-gold antibody for 2 hrs at RT. Labeled sections were washed in TBS, and contrasted with saturated uranylacetate. Samples were investigated with a transmission electron microscope (Libra 120, Zeiss, Germany) at 80 kV, and images taken with a 2048 x 2048 CCD camera (Tröndle, Moorenweis, Germany).

**Antibodies**—Rabbit anti-Nrxn (A473) (1) and anti-synapsin (E028) (62), mouse anti-Nlgn1 (4C12, Synaptic Systems, Göttingen), anti-Nlgn2 (Synaptic Systems), anti-αDAG (VIA4-1, I1H6C4, Upstate/Millipore, Billerica), anti-HA (12CA5, Roche, Mannheim, HA.11 clone 16B12, Covance, München), and a new affinity-purified rabbit anti-Nxph1 against peptide AQTV IDAKD SKSC in the variable linker between conserved domains (Eurogentec, Belgium) were used for immunolabeling.

**Structural Modeling**—SwissProt entries NRX1A_RAT (Q63372-7), NNX1H1_RAT (Q63366) and DAG1_Mouse (Q62165) were used to generate models (Figs. 3A, 4E, 6, 7C and 8A). Gas6/Axl (PDB #2CS5D) served as template for the LNS2/Nxph1 complex, in which LNS2 (PDB #2H0B) replaces Gas6. Axl residues T204 to K208 served as backbone for the β-β sheet interaction coordinates, and the Nxph1 sequence was passed in single amino acids steps through this backbone structure to generate all possible LNS2/Nxph1 complexes. Each peptide complex was scored by calculating stability with FoldX (foldx.crg.es). The complete C-terminal domain of Nxph1 was then homology modeled using PAF (PDB #2KCN) that contains an identical cysteine pattern, whereas the N-terminal domain was modeled by threading with PHYRE2 (www.sbg.bio.ic.ac.uk/phyre2). Both domains were manually connected and glycosylated using GLYCAM carbohydrate builder (glycam.ccr.columbia.edu). An alternative model of Nxph1 C-terminal domain was generated using coordinates of snake neurotoxin (PDB #1NTN). For pictograms of α DAG, the mucin region was modeled using PHYRE2 and a distorted structure as template (PDB #4A54). C-terminal domain as described (63) was modeled using N-terminal domain structure (PDB #1U2C), parts manually assembled and glycosylated with GLYCAM. For complex presentation, αDAG was manually docked to the Nrxn1α structure (PDB #3R05), while Nlgn1 and Nlgn2 dimers were placed according to co-crystals (PDB #3B3Q). All structures were rendered and visualized using PyMol (pymol.org).

**RESULTS**

αDAG, Nxph1 and α-Nrnx are expressed at inhibitory synapses—While localization of αDAG at GABAergic terminals could be demonstrated by immunocytochemistry (45,48), the hypothesized presence of Nxph1 relied on indirect evidence from in-situ-hybridization data (36) and functional deficits observed in electrophysiological recordings from knockout (KO) neurons (64).

Here, we used immunogold electron microscopy to probe the ultrastructural localization of endogenous Nxph1 in cortical tissue of adult mice. To distinguish between actual localization and residual background from a polyclonal antiserum raised against the loop region (see Experimental procedures), we compared labeling patterns in wild-type samples with KO, and performed control labelings without first antibody, using Lowicryl-embedded brain tissue. While negative controls showed essentially no labeling (data not shown), Nxph1 normally localizes specifically to membranes of symmetric, inhibitory synapses in the neocortex (Fig. 1A). Asymmetric contacts, corresponding to excitatory synapses, were not labeled (arrow in Fig. 1B), whereas Nxph1 concentrated at the synaptic cleft of symmetric profiles (arrowhead in Fig. 1B) or in compartments of the secretory pathway such as rER or Golgi cisternae (Fig. 1C), expected for a neuropeptide-like protein (35). To validate these observations, we also tested the ultrastructural distribution of its cognate receptor Nrxn and the trans-synaptic interaction partners of Nrxn, Nlgn1 and Nlgn2 in the same samples. We observed Nrxn at the synaptic cleft of both symmetric (Fig. 1D) and asymmetric (Fig. 1E) contacts in addition to localization in the secretory pathway (Fig. 1F), consistent with their widespread expression and function in inhibitory and excitatory synapses (4,43). Demonstrating the reliability of our protocol, we could confirm the subtype-specific distribution of Nlgn2 at inhibitory (Fig. 1G) and Nlgn1 (Fig. 1H) at excitatory synapses as reported (47,65). To finally brace against artifacts from our postembedding procedure that may bias
localization towards plasma membranes, we applied a pan-synapsin antibody but observed the expected different pattern over synaptic vesicles in presynaptic profiles (Fig. 1I). We conclude from our current and published results that Nxph1 is actually present at inhibitory synapses along with α-Nrxn, Nlgn2 and αDAG, providing a rationale for biochemical investigations of α-Nrxn/Nxph1-based multiplexes that might play a specific role at the GABAergic synaptic subpopulation.

αDAG binding to Nrxn—Binding of brain αDAG to α-Nrxn was reported (37) but its structural determinants and consequences for other interaction partners of Nrxn remained open. An obstacle had been the lack of information on α-Nrxn conformation, however, recent crystal data of extracellular sequences (33,66,67) allowed us to study the effects of αDAG binding based on structural predictions. The structure of α-Nrxn consists of six LNS domains (Fig. 2A, green) intercepted by three EGF-like domains (Fig. 2A, yellow) that assemble into a rigid core of LNS2-to-LNS5 (33,66). EGF2 and EGF3 show a typical ababc cysteine knot pattern that tightly join their adjacent LNS domains, while we determined here an aabbcc connectivity of EGF1 by mass spectrometry (Fig. 2A, LDE...GVC, m/z exp: 1173.81, m/z calc: 1173.44) that can open a gap between LNS1 and LNS2 by more than 11Å. Our observation explains the highly variable linkage of LNS1 (66,68) and makes LNS2 accessible. The u-form conformation of α-Nrxn (Fig. 2A) opens the possibility that binding partners of the back-folded LNS2 domain interfere with ligands at the LNS6 domain. To address this important possibility, we first determined the exact binding epitopes of αDAG and Nxph1, using a combination of co-precipitation assays and site-directed mutagenesis as previously established (20).

Building on the sole previous study on αDAG-Nrxn interaction (37), we confirmed the binding of DAG to Nrxnα and Nrxnβ, and then tested all LNS domains individually (Fig. 2B). We found that αDAG from mouse brain interacts with both LNS2 and LNS6 (Fig. 2B, upper panel). This is an interesting result because only these domains, but none of the other four LNS, were shown to mediate all ligand-binding (69), emphasizing the need to explore potentially competing complexes. Testing pulldown of Nlgn1 in the same experiments validated the known binding site at LNS6 (Fig. 2B, lane 14, middle panel) and Nrxn1β (lane 3) (15-17,20,70). In line with earlier studies which discovered that interaction of Nlgn1 and Nrxn depends on alternative splicing (25,26,71), Nlgn1 could not be pulled-down from brain lysates by full-length extracellular Nrxn1α(+SS#4) (Fig. 2B, lane 4, middle panel) or by LNS5-EGF3-LNS6 cassette with insert (Fig. 2B, lane 8, middle panel). αDAG also prefers splice insert-free LNS domains (37), and thus binds to LNS2, LNS6 and Nrxn1β without insert in SS#2 or SS#4 in our co-sedimentation assay (Fig. 2B, lanes 3, 5-6, 10, 14, upper panel). Since αDAG is able to interact with two LNS domains, it can interact with Nrxn1α(+SS#4, -SS#2) that has a blocked LNS6 but an insert-free LNS2 domain (lane 4). This is an interesting aspect as Nrxn in adult brains mostly contain (+SS#4) mRNA variants (22,72), suggesting that alternative splicing in Nrxn may affect several ligands simultaneously. While some knowledge on competitive interaction of Nlgn1 and LRRTM with Nrxn is available (42), it is unclear if αDAG also competes for the same epitope at LNS6 and how its binding to LNS2 is affected by Nxph1 (34).

To determine the site of Nxph1 binding, we had to develop a modified binding assay because normal pulldowns failed (Fig. 2C). Instead, we co-expressed recombinant Nxph1 with Fe-tagged Nrxn1 constructs in HEK293 cells, precipitated the pre-formed Nxph1/Nrxn-IgGFc complexes secreted into culture media with protein A beads, and testing binding by immunoblotting (see Experimental Procedures for details). This approach also allowed the reverse experiment with mutated Nxph1 residues, virtually impossible to accomplish by adenovirus-mediated transfer used previously to generate sufficient amounts of Nxph (34). Using the co-expression assay, we confirmed the binding of Nxph1 to isolated LNS2 (Fig. 2D, lane 6) and to the LNS1-EGF1-LNS2 cassette (lane 5), while other Nrxnα domains (lanes 4, 7-12) or Nrxn1β (lane 13) do not bind.

Interaction sites of αDAG and Nxph1 on LNS2—To study the characteristics of αDAG and Nxph1 binding epitopes on LNS2, wild-type and mutated Fe-tagged LNS2 domains were immobilized on beads, and tested for their ability to precipitate endogenous αDAG from brain lysate (Fig. 2E). As predicted from its calcium-dependency (37), αDAG binding is diminished by alanine mutations
of calcium-coordinating residues Asp-329 (lane 4, upper panel) and Gly-416 (lane 15, upper panel). More unexpectedly, nearby residues Gly-347 (lane 5, upper panel) and Tyr-412 (lane 11, upper panel) also influence binding. Since the hydroxyl group of Tyr-412 does not participate in αDAG association as indicated by mutation Y412F (lane 12, upper panel), these results suggest that hydrophobicity of these Nrxn residues is required for αDAG binding, in contrast to a basic epitope in laminin interacting with αDAG (73,74). While glycosylation of αDAG is essential for Nrxn binding (37,53), deletion of the LNS2 loop β11-β12 (residues 428-438), recently proposed to contain a carbohydrate binding site (33), demonstrates that it is not involved in αDAG binding (Fig. 2E, lane 17, upper panel). Similarly, many additional residues were tested by site-directed mutagenesis but do not influence αDAG association (lanes 6-10, 13-14 and 16 upper panel). We mapped these data to the surface of the LNS2 structure, resulting in delineation of the αDAG epitope (magenta in Fig. 2H-J).

Similar to αDAG, little is known about the Nxph1 binding epitope: Therefore, we tested the entire surface of LNS2 to determine residues required for the Nxph1/LNS2 interface. For example, we deleted distinct loops like residues 428-436 (Fig. 2G, lane 17) and changed hydrophobic to charged residues at strategic positions. We observed that the interface requires hydrophobicity of residue Ile-401 (Fig. 2F, lane 3, upper panel) but not its entire side chain (lane 8). Nearby residues, including Leu-402 (lane 4) and threonines 403-405 (lanes 7 and 9), showed no change in Nxph1 binding, when mutated to charged side chains. The only free cysteine, Cys-293 (Fig. 2I), and numerous amino acids showed no effect (Figs. 2F, lanes 6 and 8, G, lanes 4-6, and 11-16 upper panel and cyan in I-J). Since I401 is part of β10 strand in LNS2, we asked if Nxph1 engages in side chain-independent β -β interactions. We mutated the central residues of β10 to prolines and observed loss of Nxph1 complex formation (Fig. 2G, lanes 9-10), whereas binding to αDAG persisted (Fig. 2E, lanes 9-10). These data explain why binding between Nxph1 and α-Nrxn occurs calcium- and splice site-independently(34): the binding epitope at LNS2 (blue in Fig. 2H and J) is distant to these positions, and also non-overlapping with the αDAG binding site (magenta in Fig. 2H-J).

In a first attempt to assemble the Nxph1/LNS2 complex by bioinformatics, we identified the crystal structure of Gas6 LNS1/Axl as a structural template. The interaction of β-strand residues 204-208 of Axl with the correspondent β10 of Gas6 LNS1 (Fig. 2K, middle panel) is not their only contact interface (75) but suits best our purpose of modeling a Nxph1/LNS2 peptide complex (left and right panel). We generated models of all 150 possible LNS2/Nxph1 peptide combinations and calculated the relative change in free binding energy (ΔΔG, Fig.2L right panel). These results show that any sequence of 5 residues of Nxph1 will bind to β10 of LNS2, with the only exception that a tyrosine is not allowed at position 2. This restriction only limits the number of potential complexes to 140, indicating that a mutagenesis study of single positions in Nxph1 has to await more structural information.

Nxph1 prevents simultaneous binding of αDAG at LNS2—Our identification of separate binding epitopes for αDAG and Nxph1 suggested that simultaneous binding of both LNS2 ligands should be possible. We obtained complexes of Fc-tagged Nxph1 with soluble extracellular domain of Nrxn1α(+SS#4) or with LNS2-HA by co-expression that were purified and used to pulldown αDAG from neuron-like N2a cells, a rich source of endogenous αDAG. Surprisingly, the Nxph1/Nrxn1α complexes could not interact with αDAG and no triple complex was formed (Fig. 3A, lanes 7 and 8), whereas control pulldowns with Nxph1-free Fc-Nrxn1α(+SS#4) (Fig. 3A, lane 5) or Fc-LNS2 (lane 4) reliably bound αDAG. These results indicate that presence of Nxph1 may sterically constrain αDAG binding to α-Nrxn, prompting us to examine key aspects of the Nxph1 structure in α-Nrxn binding.

Based on sequence analysis (35,36), Nxph1 was identified as a preproprotein with putatively secreted mature protein consisting of glycosylated N-terminal and cysteine-rich C-terminal domains (Fig. 3B). To determine the contribution of Nxph1 domains to complex formation with α-Nrxn, we probed if N-terminal or C-terminal sequences are involved, and observed that both are required (Fig. 3C). In addition, we found that secretion of the C-terminal domain is reduced, possibly pointing to a role of the six cysteines in fold stabilization.

The highly conserved cysteines in the C-
terminal domain should help to classify its structural fold (76). However, bioinformatic prediction programs like Raptor (77), I-Tasser (78), Rossetta (79) or Phyre (80) failed to predict the cysteine connectivity or the fold. We therefore purified recombinant Fc-tagged mature Nxph1 in complex with LNS2-HA and analyzed the structure by mass spectrometry methods (81). Our co-expression system produced two secreted protein fractions, free Nxph1_Fc and Nxph1_Fc bound to LNS2-HA in a ratio of about 2:1, which we separated by gel filtration. Surprisingly, mass spectrometry revealed disulfide bonds in an abbac pattern (Fig. 4A, left and right panel), and not a more frequent cysteine knot, which is present, for example, in Nrnx1α EGF2 and EGF3 (Fig. 2A) (33,66). This connectivity was the same in free and LNS2-bound Nxph1. Since this abbac pattern is rare but might be fold stabilizing (82,83), we successively opened all bridges by cysteine to serine mutations (CS, a to a-b-c) and found that any two bridges can be opened at the same time without an effect (Fig. 4B, lanes 4-6, 10-11, 14 and 15, upper panel). However, asymmetric triple-mutations containing C239S strongly reduce Nxph1 secretion (lanes 12 and 13, lower panel). Similarly, opening of all three cysteine bridges reduce both binding capabilities (lane 7, upper panel) and secretion (lower panel), which is likely explained by a destabilized fold (84,85). The strong effect by opening the third bridge (lanes 7, 10-15) highlights Cys-239 as a key residue in fold stabilization. While these results demonstrate that Npxh1 contains a rare fold with unusual cysteine pattern required for its own secretion bound to α-Nrxn, they do not solve the question why additional binding of α DAG to preformed Nxph1/α-Nrxn is blocked.

Since the N-terminal domain of Nxph1 is also involved in complex formation (Fig. 3C, lane 5), we evaluated the contribution of N-glycosylation, its distinctive feature. N-glycosylation is not a prerequisite for complex formation as shown by tunicamycin treatment (Fig. 4C, upper panel) and point mutations of all three relevant residues including triple mutation N146D+N156D+N162D (lower panel). While this observation is consistent with earlier data (36), we now found in surface plasmon resonance (SPR) experiments with preloaded Fe-Nxph1/LNS2-HA that the glycosylation is critical for complex stability (Fig. 4D): wild-type complex bound to SPR chips resisted stringent elution (lanes 1-8, upper panel), and only near-denaturing conditions (6M urea, lane 9, 7M GdmCl, lane 10) dissolved the complex. However, the complex with non-glycosylated triple mutations already started to fall apart at 1M NaCl (Fig. 4D, 3xND, lower panel), pointing to an unexpected role of Nxph1 glycans in strengthening the interaction with α-Nrxn. We therefore analyzed the glycosylation pattern by mass spectrometry, and observed two sites occupied by complex type glycans and one by high-mannose type oligosaccharides (Fig. 4E; and table 1). While complex type glycans with terminal sialic acids and core fucose as seen here on N146 and N162 of Nxph1 are unusual for such proteins (86), we observed the same N-glycans on its cognate receptor Nrnx1α (table 1). The glycans identified add about 4kD to the N-terminal domain, leading to similar molecular weight for both domains (Fig. 3C). To visualize the complete, glycosylated mature Nxph1, we generated a model structure with glycans (Fig. 4F). The N-glycosylated N-terminal domain is shown as a single ∝ -turn (Fig. 4F, ∝1/β2, lightblue ribbon) that is flexibly linked (grey helical linker and antibody epitope) to the C-terminal domain. Since only NMR data of the antifungal protein PAF (83) described an abbac cysteine fold, we used these coordinates to model the C-terminal domain, which constitutes a three leafed 7-stranded β-fold (β3-β9, darkblue ribbon) stabilized by three cysteines (yellow sticks). In contrast to PAF, we have determined constant connectivity, but cysteine isomerization can explain the stabilizing effect of asymmetric triple-mutation CS-α+C256S (Fig. 4B, lane 14), while CS-α+C239S appears unstable (lane 12). Assuming the same cysteine cluster as in PAF, the free C239 in CS-αC256S could form an alternative cysteine bond to C194 of a, while vice versa a free C256 in CS-α+C239S is not in reach of a. From this model it is likely that the C-terminal domain will bind to β10 of LNS2 (Fig. 2J). In addition, the high-mannose type glycan on N156 is likely to be buried in the interface with α -Nrxn to protect Nxph1 from ubiquitination and ER-associated degradation (87).

Determinants of αDAG binding to α-Nrxn—While our experiments above were performed with endogenous, glycosylated dystroglycan from brain or N2a cells, purified recombinant αDAG variants
are necessary for mutagenesis. Such experiments were difficult because even large amounts of Fc-tagged αDAG secreted from HEK293 cells were hardly detectable by standard antibodies VIA4-1 or II6C4, suggesting insufficient or inappropriate glycosylation (data not shown). This situation changed with the identification of like-acetyl-glucosaminy1-trans-ferase (LARGE) that successively adds disaccharides of xylose (Xyl)-glucosamine (GlcA) terminal to complex O-mannosyl-glycans of the mucin region (Fig. 5A), which are required for αDAG binding to laminin and agrin (88,89). To test the role of LARGE for Nrxn binding, we co-transfected HEK293 cells with Fc-tagged αDAG and LARGE, and found that glycosylation of αDAG by LARGE is sufficient for binding to endogenous and recombinant Nrxn1α (Fig. 5B, lane 3, first and second panel). More importantly, using LARGE-modified recombinant αDAG we were able to pulldown α-Nrxn from mouse brain lysates (lane 3, first panel), an experiment not even reported for the intensely investigated Nlgns. While dependency of Nrxn-binding on LARGE is consistent with binding to laminin, the sites for the LARGE-mediated glycosylation appear different: we investigated by mutagenesis if Nrxn binds to the αDAG region including Thr-315 and Thr-317 that mediate laminin binding (90), but noticed that αDAG/Nrxn1α complex formation is not reduced if this region is mutated (Fig. 5C, lane 7, first and second panel).

To determine the glycosylated region of αDAG that is responsible for Nrxn-binding, we tested the complete and either half of the mucin region (Mucin, muc1 and muc2 in Fig. 5A). We observed that either half of the mucin region is sufficient to precipitate brain or recombinant α-Nrxn (Fig. 5C, lanes 4 and 5). In contrast, deletion of the complete mucin region abolished Nrxn-binding (lane 6). A control mutation of the single N-glycosylation site N139 in the N-terminal domain of αDAG had no effect (Fig. 5D, lane 4). These data indicate that binding of Nrxn to αDAG is locally less restricted compared to laminin, which binds mainly to a distinct N-terminal part of the mucin region (52,90,91).

Multiplexes of Nrxn1α—The mutually exclusive binding of Nxph1 and αDAG to LNS2 of α-Nrxn shown here indicates that multiple ligand interactions have to be considered to understand the behavior of Nrxn-based molecular complexes. Based on the u-form conformation of α-Nrxn recently identified (Fig. 2A), we asked if ligands of the LNS2 and LNS6 domains may influence each other. We found that Nxph1 in complex with full-length Nrxn1α at LNS2 prevents successive binding of αDAG because binding to its second site on the LNS6 is blocked by presence of an insert at SS#4 (Fig. 6A, lane 4). A triple complex of Nxph1/Nrxn1α/αDAG is possible, however, when the insert is missing (lane 5). Interestingly, binding of αDAG to LNS2 of Nxph1-free Nrxn1α(+SS#4) (lane 3) or to LNS6 of Nxph1/Nrxn1α(-SS#4) (lane 5) is similarly efficient, indicating an undisturbed binding of αDAG to LNS6 when Nxph1 is associated with LNS2. To brace against artifacts from a mixture of α-Nrxn with and without Nxph1 in these pulldowns, we used Fc-tagged Nxph1 for a 1:1 stoichiometry with complexed α-Nrxn. After purification, Nxph1 is present as shown by anti-Nxph1 (lanes 4-5), and the complex was used to test the binding to the third ligand αDAG (Fig. 6A, pictograms in right panel).

To address a second multiplex, we probed the binding of Nlgns to a preformed Nxph1/Nrxn1α complex (Fig. 6B). We observed that Nlgns binds normally to its sole interaction site at LNS6 independent of the presence of Nxph1 on LNS2 (Fig. 6B, lanes 3-4), resulting into a Nxph1/Nrxn1α/Nlgns triple complex (Fig. 6B, right panel). To analyze the influence of Nxph1 even on residual low-affinity binding of Nlgns to LNS6, we also included the interaction of Nlgns(+SSB) to Nrxn1α(+SS#4), a pairing of variants that can barely be detected after long incubation times (lane 6) and is even ineffective in synapse formation assays (25,31). Despite this weak interaction, the Nxph1/Nrxn1α/Nlgns triple complex could form in the presence of both splice inserts (lane 5), suggesting that interactions of Nxph1 and Nlgns with α-Nrxn occur independently of each other, in contrast to αDAG.

Finally, we examined simultaneous binding of α-Nrxn to Nlgns and αDAG. This is an important triple complex because αDAG was reported to mainly localize to inhibitory synapses (45), similar to the Nlgns variant (47) which can form a physiologically relevant trans-synaptic complex with α-Nrxn at this synapse (10,92,93). We therefore included Nlgns along with Nlgns(-B) in
our experiments, and noticed that both bind equally to Nrxn1α(+SS#4) (Fig. 6C, lane 4), consistent with a recent SPR study using an isolated LNS6 domain (71). Surprisingly, a preformed complex of Fc-tagged αDAG with Nrxn1α(+SS#4), in which αDAG can only be bound to LNS2, prevented any detectable binding of Nlgn1 or Nlgn2 (Fig. 6C, lane 3). In support, the reverse experiment with Fc-tagged Nlgn(-B) bound to Nrxn1α(+SS#4) also failed to precipitate αDAG (data not shown), suggesting that a triple complex of α-Nrxn/Nlgn/αDAG is unlikely to occur in brain. This is an unexpected result because αDAG and Nlgn compete in binding to α-Nrxn at different domains. However, it might be explained by the u-form of α-Nrxn that brings binding sites on LNS2 and LNS6 very close to each other (Fig. 2A). Thus, the relatively large and similar size of αDAG and Nlgn dimers (Fig. 6C, molecules shown in pictograms are to scale) might cause that they sterically hinder each other when bound to LNS2 and LNS6, respectively.

Binding site of αDAG on LNS6—Since the u-form conformation of α-Nrxn allows that αDAG binds simultaneously to LNS2 and LNS6, we finally analyzed the binding epitope of αDAG on the LNS6 domain. To determine the αDAG/LNS6 interface, we first confirmed the dependency of αDAG on alternative splicing in SS#4 as suggested by (37) (but see (10,25) for different results). In our experiments, αDAG binds to Nrxn1β and LNS6 without insert (-SS#4; Fig. 7A, lanes 3 and 5, first panel), and inclusion (+SS#4) completely blocks this interaction (Fig. 7A, lanes 2 and 4, first panel). Since the binding is also calcium-dependent (37), we could successfully abolish the interaction by alanine-mutations of the calcium-coordinating residues Asp-1183 (lane 9, first panel) and Gly-1201 (lane 10). As Leu-1280 and Ile-1282 were identified as hot spot residues at the interface of the Nlgn1/LNS6 complex (15-17,20,94), we introduced a triple mutation L1280S/N1284D that removes hydrophobicity from the surface surrounding the calcium-coordination site, and observed that it prevents both Nlgn1 and αDAG binding to LNS6 (lane 11). This result surprisingly indicates that hydrophobic residues are essential for αDAG. More importantly, we identified an arginine mutation of Ile-1282 that is able to discriminate between Nlgn and αDAG binding to LNS6 by blocking αDAG and leaving Nlgn1 and Nlgn2 unscathed (lane 13). These findings suggest that the more limited hydrophobicity of arginine side chains is sufficient for Nlgn association but abolishes αDAG binding. In addition to these overlapping residues, we also discovered that residue Thr-1281 is an exclusive hot spot for αDAG binding not shared by Nlgn (lane 12).

Together, our results reveal that the binding epitope for αDAG on LNS6 completely circles the calcium binding site (Fig. 7A, right panel), but also raise the question how the different epitopes of αDAG on LNS2, LNS6, and laminin LNS domains relate to each other.

To directly compare binding preferences of αDAG at the two α-Nrxn and the laminin LNS domains (51,73), we took advantage of their conserved rigid fold (20). We generated hybrid constructs of LNS2, LNS6 and LAMa2LNS5 that express swapped calcium-coordination sites, and found that the calcium-coordination of LNS6 could be transferred to LNS2 with intact αDAG binding (Fig. 7B, lane 4) but not vice versa (lane 7). The laminin calcium-coordination contains two serines (PDB #1QU0) but cannot support αDAG binding when transferred to LNS2 or LNS6 (lanes 5 and 8), even with conserved calcium-binding to such a hybrid (20). These data suggest that binding of αDAG to LNS domains of Nrxn1α is structurally different to interaction with laminin. This conclusion is supported by the fact that the entire surface of laminin LNS domains is positively charged except for the calcium binding groove (Fig. 7B, right model) and requires basic residues (51,73,74,95). In contrast, the rim surfaces of LNS2 (Fig. 7B, left model) and LNS6 (not shown) are mostly negatively charged and display hydrophobic residues near the calcium-binding groove. This unusual hydrophobic property of the Nrxn LNS domains was recognized to serve as the LNS6/Nlgn1 interface (15-17,20,70), but also mediates the important interaction of αDAG to α-Nrxn as shown above. Comparison of the two αDAG sites on LNS2 and LNS6 revealed that binding of Nxph1 might sterically hinder the approximation of αDAG to the calcium binding site (Fig. 7C, left model). Interestingly, the insert in SS#4 in switched conformation (71) of the LNS6 (middle model) is located at the same side where Nxph1 associates with LNS2, possibly mimicking a similar steric
DISCUSSION

This work presents the first biochemical and structural analysis of binding interference in α-Nrxn-based complexes, and identifies important determinants for competition in multiple interactions with Nlgn, α-DAG and Nxph.

Technical considerations—To study the α-Nrxn multiplexes, we improved methods to isolate and purify αDAG and Nxph1. First, DAG is expressed in most tissues including brain (96), but only αDAG glycosylated by LARGE is able to bind to laminins (97) and Nrxn1α (Fig. 5B). We modified the method of Sugita et al. (37) by omitting preselection with wheat-germ agglutinin (WGA) because this yielded more Nrxn1α-binding αDAG. Although WGA has been successfully applied to characterize αDAG binding to laminin (51,52,73,90,97), laminin also precipitates more αDAG without WGA (98). Second, we observed that neuroblastoma N2a cells are a rich source of Nrxn-binding αDAG which facilitated a simple lysis procedures with triton X-100 as detergent. Third, recombinant Nxph1 has previously been generated by adenovirus-mediated transfection of PC12 cell cultures (35). Here, we developed a less cumbersome, alternative strategy by co-expressing Nxph1 with Nrxn1α/LNS2 in HEK293 cells. This procedure yields a high amount of Nxph1/LNS2 complex, sufficient even for mass spectrometric analysis of glycan moities and cysteine connectivity. With these improved tools, we studied Nrxn1α forming binary and tertiary complexes with Fc-tagged αDAG, Nxph1 and Nlgn5, using a strategy that we successfully applied to determine hot spot residues at the Nrxn/Nlgn interface (20). The results from that previous biochemical investigation were entirely consistent with crystallographic studies (15-17), attesting the reliability of the current approach.

Promiscuity of LNS domains—The calcium-coordination site is described as the major binding region in LNS domains for proteins and steroid hormones (19). Our study extends its versatility to binding of glycans as determined here for αDAG/LNS2 and α DAG/LNS6. In addition, our identification of the Nxph1 binding epitope highlights β10 as a second versatile region because the receptor tyrosine kinase Axl also binds to a corresponding region at LNS1 of Gas6 (75). In support, αDAG likely covers β10 when competing with Nxph1 for binding to LNS2 (Fig. 7C), and the insert in SS#4 can replace β10 of LNS6 (71).

It is remarkable that all known, structurally diverse Nrxn ligands bind solely to the LNS2 and/or LNS6 domains. Even more astonishing is the observation that Nlgn, LRRTM and αDAG may compete for the same epitope at LNS6 (Fig. 7C). The reason may reside in an unusual calcium-induced hydrophobic and water-layered interface as shown for Nlgn1/LNS6 (15-17,20). Such hydrophobic environments are actually predicted to make stable connections to structurally diverse ligands through dynamic variations of hydrophobic contact points (99). Consistently, the conserved interfaces of Nlgn1/LNS6 (PDB #3WKF and #3B3Q) and Nlgn4/LNS6 (PDB #2XB6) differ in their hydrophobic contact points. Moreover, we show here that αDAG also requires hydrophobic residues in vicinity of the calcium binding sites of LNS2 and LNS6 (Fig. 2E and 7A).

Since Nrxn binds to complex O-mannose-type glycans that completely cover the mucin region of αDAG (Fig. 5C) (100-103), it is likely that LNS domains bind only to the accessible sugars but not to protein residues of αDAG. Accordingly, the DAG sequence is highly conserved and ligand specificity derives from post-translational glycosylation (49). We report here that Nrxn-binding depends on LARGE that adds multiple Xyl-GlcA saccharides to glycans attached to αDAG. It is still unknown how exactly sugars bind to LNS domains but hydrophobic residues are commonly found to participate in binding oligosaccharides (104,105), and their contribution for stability of a protein-glycan complex can be considerable (106). This idea is supported by crystal structures, in which mannose (PDB #1KZA) or galactose (PDB #1TLG) coordinate a calcium ion and alkyl or aromatic side chains, performing a hydrophobic stacking with the hydrophobic "bottom" of a sugar pyranose ring (104). Consistently, we determined Tyr-1281 at LNS2 (Fig. 2E) and Leu-1280 and Ile-1282 at LNS6 (Fig. 7A) as glycan interacting residues. LNS2 and LNS6 are the only LNS of α-Nrxn with a hydrophobic calcium coordination site. In
analogy, αDAG binds solely to the second LNS of pikachurin that has an phenylalanine at the position corresponding to Y412 of LNS2 in Nrxn1α (107,108). In contrast to these hydrophobic LNS domains, the binding of αDAG to laminin is mediated by basic residues on LAMA2 LG4-5 and LAMA1 LG4 (51,73,74,95). This positive surface fits to the finding that negatively charged sulfated-glycans like heparansulfate bind to laminin LNS (51,73,74,95). Furthermore, a crystal structure of LAMα2LNS5 (PDB #1QU0) revealed a sulfate ion bound to calcium and the αDAG N-terminal mucin region contains sulfated (91) in addition to phosphorylated glycans (52,109), which explains why laminin binding is restricted to this region (52,90,91). In contrast, Nrxn1α also interacts with the C-terminal mucin region (Fig. 5C) that is free of sulfated glycans (91). Together, these data allow to define two classes of αDAG binding epitopes in vicinity to calcium-coordination sites: (i) a basic surface binding to sulfated or phosphorylated glycans, and (ii) a hydrophobic surface binding directly to the pyranose ring of sugars. While laminin and agrin belong to the first class, pikachurin and Nrxn fall into the second class that is related to C-type carbohydrate sites of lectins, including the αDAG-binding concanavalin A (110).

**Multiplexes of α-Nrxn**—We tested biochemically the capability of α-Nrxn to form simultaneously complexes (‘multiplex’) with its ligands Nxph1, αDAG and Nlgn. For the purpose of a comprehensive discussion, all possible and “forbidden” multiplexes of α-Nrxns are schematically displayed in Fig. 8A. The summary shows, for example, that there are (i) no complexes with both αDAG and Nlgn, (ii) no complexes with αDAG when SS#2 and SS#4 contain inserts, and (iii) all triple complexes include Nxph1. Importantly, all Nrxn1α interactions with αDAG, Nlgns or Nxph1 investigated here represent irreversible complexes under physiological conditions. Nrxn1α bound to αDAG or Nlgn can only be disassembled by removal of calcium with EDTA (26,37), while Nxph1 can only be dissociated from Nrxn1α by near-denaturing conditions (Fig. 4D) (34,36). As a consequence, disassembly of these complexes needs stringent measures, for example, extracellular metalloproteases that have been shown to cleave βDAG (111), Nlgn1 (112) and Nrxn1α (113) from their membrane-bound C-terminus.

Under the simplistic assumption that Nrxn1α ligands are abundantly available and binding occurs randomly, the probability of complex formation is determined by the number of particular ligand complexes divided by all possible complexes. We have analyzed this probability for Nrxn1α complexes with αDAG (Fig. 8B, magenta), Nxph1 (blue) and Nlgn2 (green) at a “virtual” inhibitory terminal because these proteins are present at those synapses (45,47) (Fig. 1). Since the probability depends on the number of relevant splice sites with insert (0, 1, or 2; at SS#2 and SS#4 together), the analysis reveals that αDAG in complex with splice insert-free α-Nrxn forms with a probability of nearly 0.6 (Fig. 8B, magenta, left panel). Nlgn2 complexes (green) reach the same value when α-Nrxn contains both inserts, preventing αDAG binding. These individual probabilities of α-Nrxn complexes is a result of the competitive binding that we have biochemically determined for αDAG, Nxph1 and Nlgn. If non-competitive binding was assumed, the probability of an αDAG/α-Nrxn complex without splice inserts would be increased to about 0.8 (Fig. 8B, magenta, right panel) whereas Nlgn2/α-Nrxn complexes would not change much (green, right panel). Consequently, our analysis shows that the presence of αDAG can change the probability of Nlgn2/α-Nrxn complexes.

αDAG is expressed much earlier in development than Nrxn1α (114,115), suggesting that αDAG is an early binding partner. Our hypothesis that the probability of an αDAG/Nrxn1α complex is higher when Nrxn1α carries no splice inserts (Fig. 8B) may have important functional implications because some studies indicate that juvenile neurons express mostly insert-negative Nrxn(-SS#4) variants (116) (and unpublished observations, Irina Dudanova and M.M.). Moreover, the number of insert-positive variants appears to increase with synapse maturation (116), and +SS#4 expression is reduced after applying a learning and memory paradigm (117). In contrast to αDAG, Nlgn has a higher probability to interact with Nrxn1α containing inserts (Fig. 8B). Consistent with a role in later developmental stages, there is evidence that synaptic activity and maturation of synapses can increase the insert-
**α-Neurexin multiplexes**

positive variants via the calcium/calmodulin-dependent kinase pathway which involves RNA binding protein SAM68 (24,118). Thus, developmental and/or activity-regulated control of alternative splicing in Nrxn could modify the composition of multiplexes with αDAG, Nxph1 and Nlgn at inhibitory synapses, adding an exciting and unanticipated layer of complexity to the regulation of these essential molecules.

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**FOOTNOTES**

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**Figure Legends**

**FIGURE 1.** Ultrastructural localization of endogenous Nxph1. Immunoelectron microscopy of Lowicryl-embedded neocortical tissue from murine brain was used to determine the exact localization of Nxph1 (A-C) and its cognate receptor Nrxn (D-F). Postembedding with 10 nm gold-labeled secondary antibodies reveal Nxph1 only at symmetric, type 2 terminals (A, B, arrowheads), whereas asymmetric, type 1 contacts (B, arrows) are devoid of gold particles (circled in red in all panels). Nrxn is seen at both type 2 (D, arrowhead) and type 1 (E, arrow) synapses, representing inhibitory and excitatory terminals, respectively. (C, F) Labeling of Nxph1 and Nrxn in Golgi cisternae (Go) demonstrate their passage through the secretory pathway. (G-I) Control experiments, showing the predicted differential distribution of the trans-synaptic Nrxn ligand Nlg2 at symmetric (G) and Nlg1 at asymmetric (H) synapses. (I) A different labeling pattern is observed with anti-synapsin antibodies, confirming its association with synaptic vesicles (sv) in the presynaptic terminal of a type 1 spinous contact (sp). Scale bars, 200 nm, except in C and F, 300 nm.

**FIGURE 2.** αDAG and Nxph1 bind the same αNrxn LNS domain but different epitopes. A, modeled Nrxn1α structure in u-form conformation, modified from(33). Flexibel position of LNS1 is extended by an unusual aabbc ei disulfide pattern of the EGF1 domain (inset below) but likely constrained to an area proximal to the presynaptic membrane. Up to six alternative splice sites (SS#1-6) accept inserts (red). The stalk region between LNS6 and transmembrane region (TM) is visualized as rod-like due to massive O-glycosylation (119). B, binding of αDAG (immunoblots, upper panel) and Nlg1 (middle panel) from mouse brain was tested by pulldown with Fc-tagged extracellular domain of Nrxn1α carrying an insert in SS#4 (lane 4). Interactions of the two ligands to all six LNS domains (lanes 5,9-14), three LNS-EGF-LNS cassettes (lanes 6-8) and to Nrxn1β (lane 3) were tested, and amounts of purified Fc-tagged Nrxn domains visualized by coomassie staining (lower panel). Fc-protein was used as negative control (lanes 2 and 15). C, transfection of HEK293 cells with full-length Nxph1 produces mature protein of 25kD (lane 1) that does not bind to Fc-tagged LNS2 in pulldown assay (lane 3, immunoblot, upper panel; coomassie, lower panel). Co-expression of Nxph1 with Fc-tagged Nrxns allows complex formation and purification of Nxph1/Nrxn1α-Fc (lane 4) and Nxph1/LNS2-Fc (lane 6) from culture medium, while Nrxn1β does not bind to Nxph1 (lane 5). D, co-expression of Nxph1 with Fc-tagged Nrxn1α domains containing LNS2 yields bound Nxph1 (lanes 5-6), while other domains do not bind (lanes 4, 7-13). Co-expression of Nxph1 with β10 was used as negative control (lane 2); in cultures transfected with Nxph1 alone, the protein is hardly detectable in lysate (lane 1) and medium (lane 3). E-G, site-directed mutagenesis to identify LNS2 residues required for binding of αDAG or Nxph1. E, binding of αDAG from mouse brain lysate (lane 1) to Fc-tagged wild-type (lane 3, immunoblot, upper panel, protein staining, lower panel) or mutated (lanes 4-17) LNS2 domains was tested by pulldown. Individual mutations are described in the main text. F, binding assay after co-expression of Nxph1 with WT (lane 2) or mutated (lanes 3-9) LNS2-Fc in cell culture. G, mutations preventing αDAG binding (E) still bind to Nxph1 (lanes 4-5, 11 and 16, upper panel). Reversely, mutations of residues 1401 (lane 8, upper panel), Thr-403, Thr-404 or Thr-405 (lanes 9-10, upper panel) block Nxph1 binding. Co-expression of Nxph1 with Fc was negative (F, lane 1 and G, lane 2). H, ribbon structure of LNS2 with αDAG binding epitope (magenta) near the calcium co-ordination site and epitope for Nxph1 (blue) at β10 strand. Approximate position of SS#2 in loop β8-β9 (red). I, surface view of αDAG epitope (magenta). J, binding epitope for Nxph1 (blue); non-involved residues (cyan in H-J). K, Nxph1 site at β10 of LNS2 compares to Axl binding to LNS1 of Gas6 (PDB # 2CSD). Homology of LNS domains from Nrxn1α and Gas6 (orange, middle) allowed modeling of LNS2/Nxph1 peptide complexes (left and right). L, all possible 150 complexes were scored by difference in free binding energy (ΔΔG, right) normalized to the best binding peptide. Peptides having a Tyr at position 2 are sterically hindered and peptides with a Cys at this position gave best results (ΔΔG <0.5 kcal/M).
α-Neurexin multiplexes

FIGURE 3. Nxph1 in complex with α-Nrxn prevents binding of αDAG to LNS2. A, Pictograms of interactions probed (color-coded as labeled, splice inserts in red) and representative immunoblot. Endogenous αDAG from N2a cells binds to Fc-tagged extracellular domain of Nrxn1α (lane 5) and to isolated Fe-LNS2 (lanes 2 and 4). Preformed complexes of Fc-tagged Nxph1/Nrxn1α (lane 7) or Nxph1/LNS2-HA (lane 8) prevent αDAG binding. Protein staining after pulldown (lower panel) shows that low amounts of LNS2-Fc (~55kD) are sufficient to bind αDAG (lane 4) but LNS2-HA (25kD) in complex with Nxph1-Fc (~55kD) does not (lane 8). Comparable amounts of Nrxn1α-Fc (~220kD, lane 5) and Nrxn1β (~165kD, lane 7) were used. Isolated Nxph1-Fc does not bind to αDAG (lane 6). LNS2 and glycosylated mature Nxph1 are both about 25kD (lane 8 and Fig. 2B). B, preproteins with signal peptide (cyan) and pro-domain (grey) is cleaved to generate mature Nxph1, consisting of an N-glycosylated N-terminal (light blue) and a cysteine-rich C-terminal (dark blue) domain. C, binding of Fc-tagged Nxph1 to HA-tagged LNS2 by co-expression in COS7 cells (lane 1) requires complete mature protein (lane 2), isolated N- (lane 4) or C-terminal domains (lane 5) are not sufficient. Fc-tagged glycosylated N-terminal domain has similar size as non-glycosylated C-terminal domain. Size of molecules in A are to scale, and the modeled complexes αDAG/αNrxn and Nxph1/αNrxn generated using two criteria, (i) coverage of hot spots and (ii) maximal surface area buried (see Experimental Procedures). Note that in addition to complexes shown, other conformations are not excluded.

FIGURE 4. Structural determinants of Nxph1/α-Nrxn complex formation. A, nanoESI Q-ToF mass spectrometry of recombinant Nxph1 protein (left panel) reveals an abbcac cysteine connectivity with three bridges (scheme, right panel). B, successive Cys-to-Ser mutations (lanes 4-7), analysis of single mutations (lanes 10-11) and their combinations (lanes 12-15) identify Cys-239 as most sensitive for Nxph1 binding to LNS2 (lanes 12 and 13). C239S reduces secretion of Nxph1 when combined with cysteine bridge a (lanes 12-13), similar combinations with C256S have no effect (lanes 14-15). C, N-glycosylation of Nxph1 is not required for complex formation with LNS2. Binding of recombinant mature Nxph1 to Fc-tagged LNS2 co-expressed in COS7 cells without (lane 3) and with (lane 4) addition of tunicamycin (upper panel). Successive Asn-to-Asp mutations of all N-glycosylation sites (N146, N156 and N162) do not prevent complex formation (lanes 3-6, lower panel). D, N-glycosylation stabilizes the Nxph1/LNS2 complex. In a reversed surface plasmon resonance experiment, purified Nxph1-Fc/LNS2-HA complex covalently linked to CMD chip was tested to dissolve by serial injection of 100mM NaOAc pH4 (1), 50mM Tris pH8.9 (2), 250mM NaCl (3), 500mM NaCl (4), 1M NaCl (5), 2M NaCl (6), 4M NaCl (7), 5mM EDTA (8), 6M Urea (9) and 7M GdmCl (10). Wild-type (WT) complex is not affected by most conditions (baseline not changed, 100% binding) but can be disassembled by denaturing agents (9 and 10), serving as reference for releasable protein (0% binding). In contrast to WT (square, upper panel; black trace, lower panel), deglycosylation by the triple mutation (3xND) releases 9% of the complex (red trace, lower panel) after injection of 1M NaCl. E, glycosylation pattern of the Nxph1 N-terminal domain as determined by mass spectrometry (for details, see table 1). Residues Asn-146 and Asn-162 are bound to complex type glycans containing fucose and sialic acids, Asn-156 is linked to high-mannose glycans. F, molecular model structure of Nxph1 with glycans attached.

FIGURE 5. αDAG binding to α-Nrxn requires LARGE-mediated glycosylation of the mucin-rich region. A, domain structure of DAG with glycosylation sites tested (red). B, pulldown of α-Nrxn from mouse brain (lanes 1-4, first panel) or recombinant Nrxn1α from COS-7 lysates (second panel) using Fc-tagged αDAG secreted from HEK293 cells. αDAG was co-transfected with (lane 3) or without LARGE (lane 4); α-Nrxn bind only in the presence of glycans added by LARGE (lane 3). C, αDAG glycosylation sites essential for laminin binding (A, T315, T317; (90)) are not required for Nrxn1α; WT (lane 3) and Thr-to-Ala mutated (lane 7) αDAG bind endogenous α-Nrxn (lane 1, first panel) and recombinant Nrxn1α (second panel). Pulldown of endogenous α-Nrxn (first panel) and recombinant Nrxn1α (second panel) is prevented by absence of the complete mucin region (A) of αDAG (lane 6). Removal of half a mucin region (A, mucl or mucl2) does not abolish Nrxn1α binding (lanes 4-5). D, N-glycosylation in the N-terminal domain (A, N139) is not essential for binding (lane 4). Fc-tagged αDAG variants used in C and
$D$ were produced in presence of LARGE.

**FIGURE 6.** Restrictions in $\alpha$-Nrxn multiplexes with $\alpha$DAG, Nxph1 and Nlgns. $A$, Nrxn1$\alpha$ triple complex with Nxph1 and $\alpha$DAG depends on SS$\#4$. Preformed complexes of Fc-tagged Nxph1 and Nrxn1$\alpha$ purified from HEK293 cell media are probed for binding of $\alpha$DAG-HA from COS-7 lysate with (immunoblot, lane 4) and without (lane 5) insert at SS$\#4$. Triple complex of $\alpha$DAG/Nxph1/Nrxn1$\alpha$ only forms with LNS6(-SS$\#4$) (see also Fig. 2B; and (37)). $B$, triple complex formation of Nlgn1/Nxph1/Nrxn1$\alpha$ has no splicing restrictions. Preformed complex of Fc-tagged Nxph1 and Nrxn1$\alpha$ binds to recombinant Nlgn1 (lane 3) even with inserts in Nrxn1$\alpha$ (+SS$\#4$) and in Nlgns (lane 5, similar to Nrxn1$\alpha$ alone (lanes 4 and 6). Binding of Nrxn1$\alpha$+SS$\#4$ to Nlgn1+SSB requires long incubation and exposure times (20,25). $C$, $\alpha$DAG and Nlgns do not bind simultaneously to Nrxn1$\alpha$. Recombinant Nlgns (SS$\#B$) and Nlgn2 (upper and middle panels) bind to Fc-tagged Nrxn1$\alpha$(+SS$\#4$) (lane 4), but a preformed complex of $\alpha$DAG with Nrxn1$\alpha$ (lane 3, lower panels) inhibits binding of Nlgn1(-SSB) or Nlgn2 (lane 3). All recombinant $\alpha$DAG variants were purified from HEK293 cells co-transfected with LARGE; pictograms (A-C, right) visualize the complexes tested and key results (color-coded as labeled, splice inserts in red). For $\alpha$DAG representation, the model of (63) was extended with an elongated mucin region, and some recently determined O-linked glycans (100) added. Size of molecules (right) are to scale, and the modeled complexes were generated using two criteria, (i) coverage of hot spots and (ii) maximal surface area buried (see Experimental procedures). Note that in addition to complexes shown, other conformations are not excluded. Model of Nlgn/Nrxn1$\alpha$ modified from(33,66).

**FIGURE 7.** $\alpha$DAG competes with Nlgns for binding at Nrxn LNS6 domain. $A$, site-directed mutagenesis probing the binding epitope of $\alpha$DAG at LNS6. Presence of splice insert +SS$\#4$ in Fc-tagged Nrxn1$\beta$ (lane 2) or isolated LNS6 (lane 4), and two point mutations in LNS6 (T1281A, lane 12; I1282R, lane 13) selectively block interaction with $\alpha$DAG from brain lysate (upper panel) with unchanged Nlgn1 (second panel) and Nlgn2 (third panel) binding. Other residues at the calcium-coordination site block binding to both $\alpha$DAG and Nlgn (lanes 9-11), resulting in a partially overlapping epitope on LNS6 (magenta, right panel). $B$, $\alpha$DAG binding requires calcium-coordinating and hydrophobic residues. Brain $\alpha$DAG binds to normal LNS2 (lane 3) and a hybrid LNS with triple mutation transferring the hydrophobic calcium-coordination of LNS6 onto LNS2 (lane 4). Calcium-coordination sites transferred from LNS2 to LNS6 (lane 7) or from laminin a2LG5 to LNS2 (lane 5) or to LNS6 (lane 8) abolish $\alpha$DAG binding. The rim surface of Nrxn LNS2 domain is negatively charged (red, right panel) in contrast to laminin with more basic residues (blue, right panel). $C$, structural determinants of Nrxn-ligand interaction. Molecular modeling indicating that binding of Nxph1 at LNS2 (blue, left panel) and inclusion of splice insert at SS$\#4$ in LNS6 (red, middle panel) may have a similar structural effect by sterically inhibiting the approximation of $\alpha$DAG (right panel). In addition, binding epitopes for Nlgns (green), $\alpha$DAG (magenta) and LRRTM (orange) overlap on LNS6 (yellow) but also have exclusive residues (right panel).

**FIGURE 8.** Schematic summary of $\alpha$-Nrxn-based multiplexes at a “virtual” inhibitory synapse. $A$, Binding matrix of $\alpha$DAG (magenta), Nxph1 (blue) and Nlgns (green) forming binary and triple complexes with Nrxn1$\alpha$ (white) as determined experimentally in this study. All of these molecules are present or even enriched at inhibitory terminals (10,36,45,47) and Fig. 1). We used available crystal structures of Nrxn1$\alpha$ (PDB # 3POY, 3R05), Nlgn2 (PDB # 3BL8) and generated model structures of $\alpha$DAG without the N-terminal domain (97) and of Nxph1 to create complexes. The Nlgns dimer and $\alpha$DAG are of comparable size (radius of gyration, Rg of 40Å and 35Å, respectively) and both are as long as the rigid core unit LNS2-to-LNS5 of $\alpha$-Nrxn (Rg=37Å). Both $\alpha$DAG and Nlgn2 cover the LNS5 that may cause steric hinderance and explain the mutually exclusive binding of $\alpha$DAG and Nlgns to Nrxn1$\alpha$. $B$, Probability of Nrxn1$\alpha$ in complex with $\alpha$DAG (magenta), Nxph1 (blue) and/or Nlgns (green), calculated from the total number of possible complexes that is limited by competitive binding (left panel). $\alpha$DAG complexes appear with higher probability when Nrxn1$\alpha$ is splice insert-free, while Nlgns2 complexes increase with inclusion of inserts at SS$\#2$ or SS$\#4$. If non-competitive binding was
assumed, the splice-insert dependency of Nlgn2 and the advantage of αDAG over Nlgn2 complexes would be diminished (no competition, right panel). Size of molecules in A are to scale, and the modeled complexes were generated using two criteria, (i) coverage of hot spots and (ii) maximal surface area buried (see Experimental Procedures). Note that in addition to complexes shown, other conformations are not excluded. Model of Nlgn/Nrxn1α modified from(33,66).
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Table 1: N-Glycan Structures of Nxph1 and Nrxn1α.
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Results from nanoESI Q-Tof mass spectrometry. Glycans of a complex type were found at N146 and N162 of Nxph1, and at N125, N190 and N797 of Nrxn1α. The high-mannose type N-glycan exclusively attached to N156 was shown by analyzing wild-type and mutant proteolytic glycopeptides where N162 is inactivated by mutation to Asp. A=Gal, AN=GalNAc, GN=GlcNAc, F=Fuc, M=Man, NA=NeuAc.
Figure 1
Figure 3
Figure 4
Figure 5

A

B

C

D

\( \alpha \)-Neurexin multiplexes
Figure 6

α-Neurexin multiplexes
Figure 7

A

B

C

α-Neurexin multiplexes
Figure 8

**A**

<table>
<thead>
<tr>
<th>Nrxn1α</th>
<th>0 (−SS#2, −SS#4)</th>
<th>1 (+SS#2)</th>
<th>1 (+SS#4)</th>
<th>2 (+SS#2, +SS#4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αDAG</td>
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<td>Nph1</td>
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<tr>
<td>Nph2</td>
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</tr>
</tbody>
</table>

**B**

![Graph showing competitive and non-competitive probability of occurrence](image)

α-Neurexin multiplexes
Dystroglycan Binding to α–Neurexin Competes with Neurexophilin-1 and Neuroligin in the Brain
Carsten Reissner, Johanna Stahn, Dorothee Breuer, Martin Klose, Gottfried Pohlentz, Michael Mormann and Markus Missler

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