INDUCTION OF GABAERGIC POSTSYNAPTIC DIFFERENTIATION BY α-NEUREXINS

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β-Neurexin and neuroligin cell adhesion molecules contribute to synapse development in the brain. The longer α-neurexins function at both glutamate and GABA synapses in coupling to presynaptic calcium channels. Binding of α-neurexins to neuroligins was recently reported, but the role of the α-neurexins in synapse development has not been well studied. Here we report that in COS cell neuron coculture assays, all three α-neurexins induce clustering of the GABAergic postsynaptic scaffolding protein gephyrin and neuroligin 2, but not of the glutamatergic postsynaptic scaffolding protein PSD-95 or neuroligin 1/3/4. α-Neurexins also induce clustering of GABA_A receptor γ2 subunit. This synapse promoting activity of α-neurexins is mediated by the 6th LNS (Laminin Neurexin Sex hormone binding protein) domain and negatively modulated by upstream sequences. While inserts at splice site 4 (S4) in β-neurexins promote greater clustering activity for GABA than glutamate proteins in coculture assay, α-neurexin-specific sequences confer complete specificity for GABA proteins. We further report a developmental increase in the ratio of −S4 to +S4 forms of neurexins correlating with the onset of glutamate synaptogenesis following that of GABA synaptogenesis, and activity regulation of splicing at S4. Thus, +S4 β-neurexins and even more selectively α-neurexins may be mediators of GABAergic synaptic protein recruitment and stabilization.

Synaptic connections in the brain require precise alignment of neurotransmitter receptors on dendrites opposite transmitter release sites on axons. Neurexin and neuroligin cell adhesion molecules are thought to function in development and maintenance of the two main synapse types, excitatory glutamatergic and inhibitory GABAergic synapses (reviewed in Refs. 1,2). Neuroligins, normally present on dendrites, alone are sufficient to induce presynaptic differentiation when presented to axons of cultured neurons (1-4). Conversely, neurexins, normally present on axons (5), alone are sufficient to induce postsynaptic differentiation when presented to dendrites of cultured neurons (6,7). These coculture studies suggest that neurexins and neuroligins play some role in synapse development in vivo, perhaps in protein recruitment and stabilization of synaptic complexes. There are 4-5 neuroligins in mammals. Neuroligin 1 localizes primarily at glutamatergic synapses (8), and neuroligin 2 at GABAergic synapses (6,9). Six main neurexin isoforms are derived from 3 genes (1-3) and two promoters each (α and β) (10). The shorter β-neurexins bind neuroligins via their single LNS domain (11). Alternative splicing at multiple sites also contributes to neurexin and neuroligin diversity. In particular, the absence of the splice site 4 (S4) insert in β-neurexins and presence of the B insert in neuroligin 1 selectively promotes function at glutamatergic synapses (12-15).

The importance of these protein families for human cognition is suggested by the linkage of rare mutations in neuroligins 3 and 4 to autism and mental retardation (16,17), and the association of variations in neurexin 1 with autism (18,19). Mice lacking the major neuroligins 1/2/3 exhibit defects in inhibitory and excitatory synaptic transmission and die within 24 hr of birth (20). Selective loss of neuroligin 1 or 2 results in selective defects in glutamate or GABA synapses, respectively (21). Overexpression or knock-down of neuroligins in cultured neurons also affects synapse development, altering the function and ratio of excitatory and inhibitory synapses (22-25). A role for neuroligin in development of neuronal cholinergic synapses has also recently been found (26).

α-Neurexins terminate in the same LNS domain, transmembrane region, and intracellular
region with PDZ domain binding site as β-neurexins, but contain additionally 5 LNS domains and 3 EGF (Epidermal Growth Factor)-like domains organized into 3 modules. Analysis of triple knockout mice for α-neurexin 1/2/3, leaving expression of the β-neurexins intact, revealed a surprising function in coupling presynaptic calcium channels to synaptic vesicle exocytosis (27). α-Neurexins function in transmitter release linked to N- and P/Q-type calcium channels at central nervous system synapses, in calcium-triggered exocytosis of secretory granules in endocrine cells, and to a lesser degree contribute to efficient neuromuscular transmission (28-30). Intracellularly, α- and β-neurexins bind CASK, Mint, syntenin, and synaptotagmin (31-34). Extracellularly, α-neurexins presumably have unique binding partners to explain the calcium channel coupling phenotype that is not shared with β-neurexins. Known extracellular binding partners of α-neurexins include dystroglycan (35) and the secreted peptides neurexophilins (36), although the significance of these interactions is not well understood. Originally, it was reported that α-neurexins did not bind neuroligins (11), but while the current work was in progress it became clear that α-neurexins also bind some neuroligins (12). Neurexin-1α with or without the S4 insert binds to neuroligins 2, 3, and the minor variant of neuroligin 1 lacking a B insert but not to the major variant of neuroligin 1 containing a B insert (Fig. 3 of ref. 12). Neurexin 1α lacking the S4 insert was also reported to induce clustering of gephyrin and neuroligin 2 but not PSD-95 when presented on COS cells to dentrites of cultured hippocampal neurons (14).

We set out here to characterize the activity of α-neurexins in COS cell neuron coculture assays, to test the ability of α-neurexins to induce glutamatergic and/or GABAergic postsynaptic differentiation, in comparison with β-neurexins. We further explore the structural basis for the difference in synapse promoting activity of α- versus β-neurexins, and the regulated expression patterns of neurexins with an emphasis on regulation at the S4 splice site.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal Culture and COS Cell Coculture**

Dissociated primary hippocampal neuronal cultures were prepared from embryonic day 18 (E18) rats as previously described (37,38). Hippocampi were dissociated by trypsinization and trituration. Dissociated neurons were plated onto poly L-lysine-coated glass coverslips in 60 mm culture dishes at a density of 300,000 cells/dish and cocultured over a monolayer of glia. After 2 days, cytosine arabinoside (5 μM) was added to neuron cultures to prevent the overgrowth of glia. Cultures were maintained in serum-free MEM with N2 supplements, 0.1% ovalbumin, and 1 mM pyruvate, with replacing one-third of the media per dish once per week. Neurons were treated with 100 μM 2-amino-5-phosphonopentanoic acid (APV, Research Biochemicals) beginning on day 7.

COS-7 and HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum and transfected with Lipofectamine 2000 (Invitrogen). The flat shape of COS cells was preferred for coculture versus the rounder shape of HEK cells preferred for confocal analysis of surface association. Transfected COS cells were trypsinized 24 hours later, washed, and plated at 200,000 cells/dish onto neurons pre-grown for 8-12 days in vitro (DIV). After 24 hours of coculture, cells were fixed for 15 min in warm PBS with 4% paraformaldehyde and 4% sucrose and permeabilized with 0.25% Triton X-100. For experiments involving immunocytochemistry for neuroligins in the cocultures, cells were fixed in −20°C methanol for 10 min.

**Construction of Expression Vectors**

Neurexin-1β-CFP (+S4) was described previously (13). To generate neurexin-1α-CFP, the N-terminal portion of rat neurexin 1α was cloned by RT-PCR and joined with the C-terminal portion of the mouse cDNA (BC047146; Open Biosystems) at the internal BstEII site and inserted in frame into the pECFP-N1 vector (Clontech). Neurexin 2α (AK129239) and neurexin 3α (BC060719) were first corrected for apparent errors and then cloned into pECFP-N1. For neurexin 2α, the Stratagene Site-Directed Mutagenesis kit was used to restore CAG in place of a premature TAG at residue 1301. For neurexin 3α, the splice site 1 insert consisting of an apparent duplication of exons 2 and 3 was removed by an overlap PCR method. To generate neurexin 2β’ and 3β’, the LNS domain of neurexin 1β (residues 84 to 261) was replaced with the equivalent residues of LNS6 of neurexin 2α or 3α, altering the junctional amino acids GT to EF, and EV to ST, to facilitate cloning. Neurexin 1αBC contained a deletion of residues 31-473, and neurexin 1αC a deletion of residues 31-899 (numbering according to (39), each with addition of an extra LV at the junction. All neurexin
variants used in this paper have the insert at the splice site 4 position.

**Immunocytochemistry and Imaging**

Fixed neuron/COS cell cocultures were blocked with 10% BSA (30 min; 37°C), incubated with appropriate primary antibodies in PBS with 3% BSA (overnight; room temperature), and then with secondary antibodies (45 min; 37°C). Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1, 4-diazabicyclo [2, 2, 2] octane). For Figs. 1-2, cocultures were stained with anti-gephyrin (mAb7a; IgG1; 1:500; Synaptic Systems), anti-PSD-95 (6G6-1C9; IgG2a; 1:500; Affinity Bioreagents), and anti-synapsin (rabbit; 1:1000; Chemicon) followed by secondary antibodies (45 min; 37°C). Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1, 4-diazabicyclo [2, 2, 2] octane). For Figs. 1-2, cocultures were stained with 2% 1, 4-diazabicyclo [2, 2, 2] octane). For Figs. 1-2, cocultures were stained with anti-gephyrin (mAb7a; IgG1; 1:500; Synaptic Systems), anti-PSD-95 (6G6-1C9; IgG2a; 1:500; Affinity Bioreagents), and anti-synapsin (rabbit; 1:1000; Chemicon) followed by secondary antibodies conjugated to Alexa 488, Alexa 568, and Alexa 647 (Molecular Probes), respectively. For Fig. 4, cocultures were labeled for neuroligin-1/3/4 (Alexa 647; Molecular Probes), and anti-synapsin (rabbit; 1:1000; Chemicon) followed by secondary antibodies conjugated to Alexa 488, Alexa 568, and Alexa 647 (Molecular Probes), respectively.

For quantification for Figs. 3 and 4, images were randomized so that the experimenter was blind to the transfection group. The area for measuring was defined by the perimeter of the transfected COS cell. Images of the presynaptic and postsynaptic proteins were thresholded. For each postsynaptic protein cluster, a region was drawn around each cluster and the area and total gray value was measured. Thresholded synapsin was measured through postsynaptic protein regions to determine which clusters were synaptic. Postsynaptic protein clusters that were apposed to synapsin were excluded from the final quantification. Analysis was performed using MetaMorph and Microsoft Excel. All data are reported as mean ± SEM.

For quantification for Fig. 6, cocultures were assessed blind to the transfection group. On each coverslip, all neurexin-CFP transfected COS cells having significant contact with neuronal dendrites based on phase contrast were assessed (up to 40 cells per coverslip). The area associated with each chosen COS cell was then visually scored as either positive or negative, positive indicating the presence of any clusters of postsynaptic protein (PSD-95, gephyrin or neuroligin 2) that lacked associated staining for synapsin.

**RNA Isolation and RT-PCR**

For RNA extraction from brain, either two hippocampi or roughly 50 mg of cortex were dissected from rats at embryonic day 18 (E18), postnatal day 11 (P11) or adult (Ad), rapidly homogenized in 1 ml of TRizol reagent (Invitrogen), and RNA prepared according to the manufacturer’s protocol. For RNA extraction from hippocampal cultures, roughly 1 x 10^6 cells that were either untreated or chronically treated with 100 µM APV were harvested by briefly washing in cold PBS and either scraping directly in TRizol reagent or by using the RNasy Easy Mini Kit (Qiagen) according to the manufacturer’s protocol. First strand cDNA synthesis was performed on 3 µg total RNA for brain tissue samples or 300 ng total RNA for hippocampal culture samples in a total volume of 50 µl using 500 Units of SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer. Extension of cDNA was performed at 50°C for 60
minutes. The cDNA was then treated with 4 units of Ribonuclease H at 37°C for 30 minutes. PCR amplification was carried out on 0.5 μl of each above cDNA template in a total volume of 20 μl using 1 Unit of Taq DNA Polymerase (Invitrogen). Thermal cycling parameters were as follows: denaturation at 95°C for 30 seconds; annealing at 57°C for 30 seconds; extension at 72°C for 40 seconds. PCR was performed for 25-40 cycles and the entire reaction loaded on 2% agarose gel for electrophoresis. Following staining and visualization with ethidium bromide, semi-quantitation of band intensities was performed using ImageJ software. Two independent RNA preparations were made from independent animals or hippocampal cultures and RT-PCR was performed in triplicate. All data are reported as mean ± SEM.

Primers for each neurexin isoform were designed to include one reverse priming site downstream of splice site 4 that would be common to both the α and β isoforms and a unique forward priming site upstream of splice site 4 that would be exclusive to either the α or β isoform. The primers used in this study were (5’ to 3’): Nrxn1αF, ATGGGATGGCTTTAGCTGTG; Nrxn1βF, GGTCACCAGCATTCTTGC; Nrxn1R, CCCGCAAATTATTATGGTGC; Nrxn2αF, ACTTCCATAGGAGGCTGT; Nrxn2βF, CCACCACGTCCACACTT; Nrxn3αF, TGGCTGTTGAAGATGGTCAG; Nrxn3βF, CCACCACGTCCACTT; Nrxn3R, AGAGAGTTGGCCTTGGAAGA; GAPDH-F, TCTACACCTGGCCAGCAAAT; Nrxn3αβ, ACGATGCTCTCCACAGGAGT; Nrxn3βα, TGGCTGTTGAAGATGGTCAG; Nrxn2R, ACTTCCTATGGAGGCCCTGT; Nrxn2β, CCCACCACGTCCACCACTT; Nrxn1R, β, ATGGGATGGCTTTAGCTGTG; Nrxn1α, 2 or β-neurexins, mediates binding to specific neuroligins (12). Thus for comparison, we assayed in the same experiments the sixth LNS domain of neurexin 1α, 2α, and 3α each in the context of the neurexin 1β flanking sequences, corresponding to neurexin 1β itself, and constructs termed neurexin 2β' and neurexin 3β' (similar to neurexins 2β and 3β but with the regions flanking the LNS domain derived from 1β; Figs. 2, S2). We expressed the neurexin variants tagged intracellularly with cyan fluorescent protein (CFP) in COS cells, cocultured with hippocampal neurons, and assayed for ability to induce postsynaptic differentiation (as in refs. 6,13). The ability of each neurexin to induce clustering of the inhibitory postsynaptic scaffolding protein gephyrin or the excitatory postsynaptic scaffolding protein PSD-95 in contacting dendrites was determined. Co-labeling for the presynaptic marker synapsin was used to identify the few endogenous inter-neuronal synaptic clusters that may underlie the transfected COS cells since these bona fide synapses label for PSD-95 or gephyrin plus synapsin. Additional clusters of PSD-95 or gephyrin lacking apposed synapsin immunofluorescence were specifically associated with COS cells expressing neurexins, and are referred to as 'induced clusters' throughout this paper. Such non-synaptic clusters of PSD-95 or gephyrin were not observed associated with control COS cells expressing membrane-associated CFP (mCFP; Fig. 1B). Induced clusters of PSD-95 or gephyrin were frequently but not always localized near the edges of neurexin-expressing COS cells, presumably where the COS cells come into closest contact with the neighboring neurons. Most often, the inducing neurexin was highly expressed over the entire COS cell and not visibly clustered, although rarely neurexin clusters on the COS cell were observed associated with dendritic protein clusters (e.g. Fig. 3 of ref. 6).

All three α-neurexins induced prominent clustering of gephyrin but no detectable clustering of PSD-95 in contacting dendrites (Figs. 1, S1). In contrast, like neurexin 1β (13), neurexin 2β' and 3β' induced prominent clustering of both gephyrin and PSD-95 (Figs. 2, S2). While the induced clusters of gephyrin and PSD-95 appear to overlap at low resolution, at higher resolution they usually resolve into separate clusters side by side (Fig. S2 and ref. 6). Quantitation of clusters of PSD-95 or gephyrin lacking synapsin was performed on random sets of COS cells expressing each neurexin variant in comparison with the baseline clusters associated with COS cells expressing only mCFP. This quantitation confirmed a significant induction of PSD-95 clusters only by the three β-neurexin constructs but none of the α-neurexins, and induction of gephyrin clusters by all neurexin variants tested (Fig. 3, p <0.05 or p<0.005 as
indicated, n=32-35 cells each). For induction of gephyrin clustering, the α-neurexins exhibited weaker activity than the β-neurexin constructs, but were still 2- to 7-fold above the background of mCFP. In this same set of experiments showing differences in ability to induce postsynaptic protein clustering, expression levels per COS cell did not differ among constructs (average relative CFP intensity values for each neurexin were 92 for 1α-CFP, 103 for 1β-CFP, 85 for 2α-CFP, 97 for 2β-CFP, 113 for 3α-CFP, 110 for 3β'-CFP; ANOVA p>0.1).

The same type of coculture assay can be used to test the recruitment of neuroligin 1/3/4 (using an antibody that recognizes a common epitope) versus neuroligin 2 to contact sites of dendrites with neurexin-expressing COS cells. Neuroligin clusters induced by neurexin contact were again differentiated from endogenous interneuronal synaptic clusters by the absence of associated synapsin immunoreactivity. We again observed a differential activity between α versus β neurexins. Although the area of induced clusters of the neuroligins tends to be larger than that of gephyrin or PSD-95, covering a larger contact zone rather than being split into clusters more typical of a synaptic size (Ref. 40 and compare Figs. 1, 2 and 4), we could detect no significant clustering of neuroligin 1/3/4 by the α-neurexins (Fig. 4). All three α-neurexins induced prominent clustering of neuroligin 2 but not neuroligin 1/3/4 in contacting dendrites. In contrast, like neurexin 1β (13), neuroligin 2β' and 3β' induced prominent clustering of both neuroligin 2 and neuroligin 1/3/4. While this neuroligin 1/3/4 antibody recognizes all three neuroligins (41), neuroligins 1 and 3 are present at similar levels but neuroligin 4 is not detectable in neonatal rodent tissue (20). Furthermore, neuroligin 1 is mainly expressed in the +B splice form (14) and neuroligin 1 +B binds neuroligin 1β with 2-6-fold greater affinity than neuroligin 3 (15). Thus the major variant clustered by β-neurexins but not α-neurexins and recognized by the neuroligin 1/3/4 antibody in this study is likely neuroligin 1 +B.

Finally, we tested for the ability of each neurexin construct to induce clustering of the two other major components of GABAergic synapses, GABA<sub>λ</sub> receptors and dystroglycan. The major synaptic GABA<sub>λ</sub> receptor subunit γ2 (42), like gephyrin, was clustered by all α-neurexins and β-neurexins tested. One example of induced clustering of GABA<sub>λ</sub> receptor γ2 is shown for neurexin 2α in Fig. 5. Qualitatively, the relative clustering ability of the different constructs for GABA receptor γ2 appeared to parallel that for gephyrin (data not shown). Unlike GABA<sub>λ</sub> receptor γ2 which is mainly synaptic, GABA<sub>λ</sub> receptor α5 subunit is mainly extrasynaptic, contributing to tonic GABAergic signaling (40,43). In a coculture experiment of neurons with COS cells expressing neurexin2α-CFP, immunostaining of sister coverslips revealed obvious induced nonsynaptic clustering of GABA<sub>λ</sub> receptor γ2 but no detectable clustering of GABA<sub>λ</sub> receptor α5 (Fig. S3). As previously described, endogenous GABA<sub>λ</sub> receptor α5 was detected more diffusely along dendrites compared with γ2 which was clustered at synapses. Some inhomogeneity in α5 immunoreactivity was observed along many dendrites, but at sites unrelated to COS cell contacts, and strong clusters were never observed. Thus the effects of neurexins are selective for clustering GABA<sub>λ</sub> receptor γ2 but not α5 subunit. Since Fig. S3 was performed with live cell primary antibody incubation, this also shows that the α-neurexin-induced clusters of GABA<sub>λ</sub> receptor γ2 are on the dendrite surface. Dystroglycan is present at only a subset of mature GABA synapses (44,45), but was also reported to bind directly to α-neurexins (35). However, in the coculture assay, we did not observe clear induced clustering of dystroglycan by any neurexin, α or β, on neurons at 2-4 weeks in culture.

**Structural Basis for the Differential Synaptogenic Activity of α-Neurexins Versus β-Neurexins**

The C-terminal region of neurexin-1α is identical to that of neurexin-1β from LNS6 on, and yet we show here that the synapse promoting activity of neurexin 1α is weaker and more specific for GABAergic postsynaptic proteins than that of neurexin 1β in the coculture assay. Previous work has shown that a construct containing only the region common to neurexin 1α and 1β (deleting the β-specific sequence from neurexin 1β) had postsynaptic clustering activity equal to that of neurexin 1β (6). Thus, the difference in synaptogenic activity between neurexin 1α and 1β is due to an inhibitory effect of α-specific sequences rather than an enhancing effect of β-specific sequences. To explore the basis of this differential activity, we made sequential deletions from the mature N-terminus of neurexin 1α, leaving the signal sequence for proper surface expression (Fig. 6). All deletion and mutant neurexin-CFP constructs assayed in this paper appeared to reach the cell surface as efficiently as neurexin 1α and neurexin 1β, as assayed by
confocal optical sections through transfected HEK293 cells (Fig. S4). Furthermore, expression level did not vary significantly among neurexin-CFP constructs in the COS cell expression studies (average relative CFP intensity values for each neurexin were 111 for 1α-CFP, 95 for 1αBC-CFP, 102 for 1αC-CFP, 103 for 1αD1176A-CFP, 89 for 1β-CFP; n=15-21, ANOVA p>0.1).

Deleting module A containing LNS1, LNS2 and the intervening EGF-like domain had no effect; the ability of this construct termed neurexin 1αBC to cluster postsynaptic proteins was indistinguishable from that of full-length neurexin 1α (Fig. 6A,B). In contrast, further deleting module B containing LNS3, LNS4 and the intervening EGF-like domain resulted in increased synapse promoting activity. The ability of this construct, termed neurexin 1αC, to cluster GABAergic postsynaptic proteins gephyrin and neuroligin 2 was intermediate between that of neurexin-1α and neurexin-1β. However, while deletion of modules A and B together significantly increased clustering of gephyrin and neuroligin 2 compared with full length neurexin 1α, it did not increase clustering of PSD-95 (Fig. 6). Thus LNS5 and/or the third EGF-like domain within module C apparently inhibit the ability of neurexin 1 to cluster PSD-95, contributing synapse-type specificity.

Boucard et al. (12) found that LNS6 was the only LNS domain of neurexin 1α able to bind neuroligin when tested individually or in modules. We showed previously that a single point mutation to a predicted calcium-binding residue D137A of this LNS domain in the context of neurexin 1β abolishes binding to neuroligin and abolishes synaptogenic activity (13). We thus tested whether this single point mutation in LNS6 in the context of full-length neurexin (i.e. 1α D1176A) affects synaptogenic activity. Indeed, neurexin 1α D1176A completely lacked the ability to cluster postsynaptic proteins (Fig. 6B). Thus LNS6 is essential for mediating postsynaptic protein clustering in neurexin 1α, and the upstream sequences in modules B and C, regardless of additional LNS domains, inhibit rather than enhance the synaptogenic activity of neurexin 1α.

**Expression Patterns of Neurexins**

Neurexin expression is brain-specific (39). At the cellular level, the six major neurexin forms 1-3 α and β show fairly broad overlapping expression patterns in brain, such that most neurons express multiple neurexins (46). How the expression patterns change with development has not been reported for mammalian central nervous system. We addressed this question here, with emphasis on the S4 insert given its importance in regulating the synaptogenic activity of β-neurexins (12-14). By RT-PCR, we could readily detect all six neurexin forms 1-3 α and β from E18 to adult in rat hippocampus and cortex (Fig. 7A). There was a consistent increase in the percentage of all neurexins lacking the S4 insert versus containing the S4 insert through development in both hippocampus and cortex. The difference in ratio of splice variants was greatest for neurexin 3 α and β, for which the percent lacking the S4 insert changed from <5% at E18 to 35-50% at P11 (Fig. 7B). Neurons from E18 rat grown in culture for 7-22 DIV showed a similar trend with the percent of neurexins lacking the S4 insert increasing with development (Fig. 8).

To assess potential activity regulation, we grew hippocampal neurons in culture under control conditions or chronically in the presence of the NMDA receptor antagonist APV. Semi-quantitative RT-PCR in comparison to GAPDH revealed no significant difference in total level of each neurexin mRNA (-S4 and +S4 forms combined) due to the presence or absence of APV (data not shown). However, blockade significantly increased the percentage of neurexin 2β lacking the S4 insert (from ~20% to ~40%), while reducing the percentage of neurexins 3α and 3β lacking the S4 insert (Fig. 8B). Thus NMDA receptor activity differentially regulates splicing of neurexins at the S4 splice site.

**DISCUSSION**

We show here that α-neurexins promote GABAergic but not glutamatergic postsynaptic specialization. Specifically, α-neurexins expressed on COS cells cluster gephyrin and GABA_A receptor, but have no detectable effect on the distribution of PSD-95 in contacting dendrites of cultured hippocampal neurons (Figs. 1-3, 5). This specificity presumably arises at least in part from the specific clustering of neuroligin 2 but not neuroligins 1/3/4 on the contacting dendrites by α-neurexins (Fig. 4). The postsynaptic clustering activity of α-neurexins is dependent on LNS6, being abolished by a single point mutation in the predicted calcium-binding residue D1176A (Fig. 6). The central region of α-neurexin containing LNS 3-5 and two EGF-like domains inhibits the synaptogenic activity of LNS6 in the context of full-length α-neurexin (Fig. 5). Finally, we report
that splicing of all neurexins at the S4 site is developmentally regulated, with levels of neurexins lacking the S4 insert low just before birth and increasing during the first two postnatal weeks in rat cortex and hippocampus (Fig. 7). Major differences between neurexins 1, 2, and 3 were not observed in these assays except for activity regulation of splicing at the S4 site, with differential regulation in 2β versus 3α,β (Fig. 8).

A major conclusion from this study is that α-neurexins are completely specific for promoting GABA but not glutamate postsynaptic differentiation in coculture assays. β-Neurexins containing the S4 insert were also previously shown to be more active for promoting GABA than glutamate postsynaptic differentiation in coculture assays (13,14). Chih et al. (14) did not observe induction of PSD-95 clusters by either neurexin 1β containing the S4 insert or neurexin 1α lacking the S4 insert, and suggested that α-neurexins have a similar GABAergic specificity for synaptogenic activity as β-neurexins containing the S4 insert. However, we show here and in Graf et al. (13) that neurexin 1β containing the S4 insert does cluster PSD-95 significantly above the background level assessed by mCFP expression in COS cells. In the same experiment where we observe significant clustering of PSD-95 by all β-type neurexin constructs (Fig. 1, all +S4 variants), we find no significant clustering of PSD-95 by α-neurexins. It is possible that we have observed these more subtle distinctions due to differences in experimental conditions from Chih et al. (14), including differences in construct expression level or neuron culture style. In experiments where we tried to bias towards seeing any effect of α-neurexins on distribution of glutamatergic postsynaptic proteins, assaying the more strongly clustered neuroligins rather than the scaffolding proteins, we could still find no effect of α-neurexins on distribution of neuroligin 1/3/4 (Fig. 4, noting that the major variant recognized by the common antibody in this experiment is thought to be neuroligin 1 +B as detailed in the results section above). In the same experiment, the α-neurexins could induce robust clustering of neuroligin 2 (Fig. 4). Previous evidence shows that neuroligin 2 is normally clustered only at GABA synapses (9,13) and neuroligin 1 primarily at glutamate synapses (8). Subcellular localization of endogenous neuroligins 3 and 4 has not been reported, but the distribution of YFP-tagged forms of neuroligins 3 and 4 is similar to that of neuroligin 1 and not 2 (6). We conclude here that α-neurexins induce clustering of the GABAergic proteins gephyrin, GABA_A receptor and neuroligin 2, but not of the glutamatergic protein PSD-95 or neuroligin 1/3/4.

These differences in ability to cluster specific postsynaptic components agree well with the binding selectivity of neurexins to neuroligin splice variants as reported recently (12). The majority of neuroligin 1 in the brain is present in the form with the B insert (14). β-Neurexins with the S4 insert have lower binding affinity for neuroligin 1 with the B insert than without the B insert (12-14). However, this difference is not an absolute effect, since in the most sensitive binding assay incubating soluble neurexin forms to neuroligins expressed on the surface of HEK293 or COS cells, β-neurexins with the S4 insert still bound specifically to cells expressing neuroligin 1 with the B insert compared with untransfected control cells or compared with binding of an LNS deletion variant of neuroligin (12,13). In contrast, in the same experiment binding of α-neurexins with or without the S4 insert to neuroligin 1 with the B insert was not detected (12). In short, all β-neurexins bind neuroligin 1 +B but α-neurexins do not. Thus, we conclude that the binding to neuroligin 1 with the B insert and ability to cluster glutamatergic postsynaptic proteins in coculture occurs in parallel. Furthermore, addition of the S4 insert to β-neurexins reduces but does not abolish these activities, whereas the presence of the additional sequences in the longer α-neurexins abolishes these activities.

Regulation of neurexin splicing has not yet been rigorously studied, although differential regulation can occur among mammalian brain regions (46) and during development in other systems (47,48). Specifically with respect to the S4 site, differences in ratios of +S4 versus -S4 variants of selected α- or combined α- plus β-neurexins have been reported among adult rat brain regions (49) and during development and neurotrophin exposure in chick sympathetic neurons (47). Here we show that the percentage of each α and β-neurexin lacking the S4 insert increases between E18 and P11 in rat cortex and hippocampus. This developmental increase in the more glutamate-selective -S4 β variants correlates with the developmental increase in glutamatergic synaptogenesis. The earlier expression of the more GABA-selective +S4 β variants correlates with the earlier onset of GABAergic synaptogenesis (50,51). Neurexin 3 α and β, which showed the greatest developmental regulation, showed a similar increase in -S4 variants with development of E18
hippocampal neurons in dissociated culture from DIV 7 to 14 (Fig. 7). Interestingly, in the culture system where we could manipulate activity, we found that NMDA receptor blockade differentially regulates splicing, increasing -S4 variants of 3α,β and decreasing -S4 variants of 2β. The significance of this differential activity regulation is not yet clear but may be important in combination with regulation of splicing at site 5 which is predicted to generate secreted forms of neurexin 3 but not 1 or 2 (52). No obvious developmental changes were observed for overall expression levels of α-neurexins, perhaps reflecting more their calcium channel coupling function at many synapse types rather than their additional GABA-specific roles.

Analysis here of the single point mutation D1176A in the predicted calcium binding residue of LNS6 indicates that LNS6 is necessary for GABAergic synaptogenic activity of neurexin-1α, no other LNS domains are active (Fig. 6). Again, this coculture result is in good agreement with the binding data of Boucard et al. (12). These authors found that LNS6 and a short upstream region is the minimal sequence necessary and sufficient from α-neurexin for binding neuroligin lacking the B insert. The combination of this published binding data and our coculture studies strongly suggest that α-neurexins cluster gephyrin and GABA_α receptor through clustering neuroligin 2 on dendrites, via binding of neuroligin 2 to LNS6. While the α-neurexin specific region upstream of LNS6 presumably interacts with other proteins and functions in coupling presynaptic calcium channels to release (28), our deletion analyses show that this region of α-neurexin inhibits the synaptogenic activity of the C-terminal portion containing LNS6 (Fig. 6). The inhibitory activity did not reside in module A but in modules B and C. These upstream regions may either participate in an intra-molecular interaction inhibiting the activity of LNS6, or may bind an additional inhibitory factor.

More generally, in the coculture assays, all neurexins induce clustering of GABAergic proteins to the same or a greater extent than that of glutamatergic proteins (e.g. comparing increase in integrated intensity relative to the background on mCFP cells (Fig. 3) or comparing total area of induced clusters since non-normalized intensity would not be directly comparable (13,14)). This equal or greater activity for promoting GABA postsynaptic development applies to all neurexins tested to date, 1-3, α and β, and containing or lacking the S4 insert. While all splice forms of α-neurexins have not been tested, given that LNS6 of the α-neurexins bears the synaptogenic activity, it is highly unlikely that any forms not yet tested would have stronger activity for inducing glutamatergic than GABAergic postsynaptic protein clustering. These data are consistent with the idea that the neurexin-neuroligin system may be more important for the development and maintenance of GABA synapses than glutamate synapses. Support for this idea comes from the finding that inhibitory synaptic transmission is more severely impaired than excitatory synaptic transmission in P0 brainstem of the neuroligin 1/2/3 triple knockout mice (20). Spontaneous glutamatergic transmission frequency was reduced 4-fold and evoked failure rate unaffected, whereas spontaneous GABAergic/glycinergic transmission frequency was reduced 10-fold and evoked failure rate markedly increased. At the same time, survival of the individual neuroligin knockouts but neonatal death of the triple knockout (20) indicates some redundancy in function rather than an absolute requirement for neuroligin 2 function at GABA synapses. In addition to the calcium channel coupling phenotype, α-neurexin 1/2/3 triple knockout mice had a twofold reduction in the density of type II symmetric GABAergic synapses in neonatal brainstem with no change in density of type I asymmetric glutamatergic synapses (27). A 30% reduction in type II symmetric synapses but not type I asymmetric synapses was also found in adult neocortex of neurexin 1/2α and 2/3α double knockout mice (53). It was not clear whether this loss of inhibitory synapses reflected a direct function of α-neurexin in GABA synapse development or an indirect effect of reduced synaptic activity due to the presynaptic calcium channel coupling defect. Based on the coculture results presented here, we suggest that the loss of inhibitory synapses in these mice may reflect a direct function of α-neurexins in linking GABAergic presynaptic and postsynaptic components. The apparent requirement for α-neurexin LNS6 to bind neuroligins for coculture activity (D1176A in Fig. 6 combined with (12,13)) suggests that the function of α-neurexins in GABA synapse formation and/or stabilization occurs via binding neuroligins. Although we could not observe clustering of dystroglycan by neurexins in the coculture assay, and GABA synapses can form in the absence of dystroglycan (45,54), the association of α-neurexins with dystroglycan (35) may also contribute to stabilization of a postsynaptic complex at some GABA synapses (44). Members of the SynCAM, ephrin and Eph
receptor, and netrin G ligand families are reported to trigger presynaptic and/or postsynaptic differentiation specifically for glutamate and not GABA synapses (55-58). The stronger functional association of the neurexin-neuroligin complex with GABA compared with glutamate synapses in coculture and in the knockout mice may reflect a greater redundancy of synaptic organizing molecules at glutamate synapses. In Drosophila, which lack β neurexins and have less redundancy in general, null mutations in the single α neurexin gene lead to a reduction in numbers of central and neuromuscular synapses, altered ultrastructure, and defects in synaptic transmission and associative learning (59,60).

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ABBREVIATIONS
GABA, γ-aminobutyric acid
LNS, Laminin Neurexin Sex hormone binding protein
S4, splice site 4
EGF, Epidermal Growth Factor
MEM, minimal essential medium
APV, 2-amino-5-phosphonopentanoic acid
DIV, days in vitro
PBS, phosphate buffered saline
CFP, cyan fluorescent protein
BSA, bovine serum albumen
NMDA, N-methyl-D-aspartate
E18, embryonic day 18
P11, postnatal day 11
GAD, glutamic acid decarboxylase
GAPDH, glyceraldehyde phosphate dehydrogenase

REFERENCES
1. Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000) Cell 101, 657-669
2. Fu, Z., Washbourne, P., Ortinski, P., and Vicini, S. (2003) J Neurophysiol 90, 3950-3957
3. Chubykin, A. A., Liu, X., Comoletti, D., Tsigelny, I., Taylor, P., and Sudhof, T. C. (2005) J Biol Chem 280, 22365-22374
4. Dong, N., Qi, J., and Chen, G. (2007) Mol Cell Neurosci 35,14-23.
5. Berninghausen, O., Rahman, M. A., Silva, J. P., Davletov, B., Hopkins, C., and Ushkaryov, Y. A. (2007) J Neurochem
6. Graf, E. R., Zhang, X., Jin, S. X., Linhoff, M. W., and Craig, A. M. (2004) Cell 119, 1013-1026
7. Nam, C. I., and Chen, L. (2005) Proc Natl Acad Sci U S A 102, 6137-6142
8. Song, J. Y., Ichtchenko, K., Sudhof, T. C., and Brose, N. (1999) Proc Natl Acad Sci U S A 96, 1100-1105
9. Varoqueaux, F., Jamain, S., and Brose, N. (2004) Eur J Cell Biol 83, 449-456
10. Tabuchi, K., and Sudhof, T. C. (2002) Genomics 79, 849-859.
11. Ichtchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Sudhof, T. C. (1995) Cell 81, 435-443.
12. Boucard, A. A., Chubykin, A. A., Comoletti, D., Taylor, P., and Sudhof, T. C. (2005) Neuron 48, 229-236
13. Graf, E. R., Kang, Y., Hauner, A. M., and Craig, A. M. (2006) J Neurosci 26, 4256-4265
14. Chih, B., Gollan, L., and Scheiffele, P. (2006) Neuron 51, 171-178
15. Comoletti, D., Flynn, R. E., Boucard, A. A., Demeler, B., Schirf, V., Shi, J., Jennings, L. L., Newlin, H. R., Sudhof, T. C., and Taylor, P. (2006) Biochemistry 45, 12816-12827
16. Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I. C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C., and Bourgeron, T. (2003) Nat Genet 34, 27-29.
17. Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M. P., Raynaud, M., Ronci, N., Lemmonier, E., Calvas, P., Laudier, B., Chelly, J., Fryns, J. P., Ropers, H. H., Hamel, B. C., Andres, C., Barthelemy, C., Moraine, C., and Briault, S. (2004) Am J Hum Genet 74, 552-557
18. Feng, J., Schroer, R., Yan, J., Song, W., Yang, C., Bockholt, A., Cook, E. H., Jr., Skinner, C., Schwartz, C. E., and Sommer, S. S. (2006) Neurosci Lett 409, 10-13
19. Szatmari, P., Paterson, A. D., Zwaigenbaum, L., Roberts, W., Brian, J., Liu, X. Q., Vincent, J. B., Skau, J. L., Thompson, A. P., Senman, L., Feuk, L., Qian, C., Bryson, S. W. E., Jones, M. B., Marshall, C. R., Scherer, S. W., Vieland, V. J., Bartlett, C., Mangin, L. V., Goedken, R., Segre, A., Pericak-Vance, M. A., Cuccaro, M. L., Gilbert, J. R., Wright, H. H., Abramson, R. K., Betancur, C., Bourgeron, T., Gillberg, C., Leboyer, M., Buxbaum, J. D., Davis, K. L., Hollander, E., Silverman, J. M., Hallmayer, J., Lottspeich, L., Sutcliffe, J. S., Haines, J. L., Folstein, S. E., Piven, J., Wassink, T. H., Sheffield, V., Geschwind, D. H., Bucan, M., Brown, W. T., Cantor, R. M., Constantino, J. N., Gilliam, T. C., Herbert, M., Lajonchere, C., Ledbetter, D. H., Lese-Martin, C., Miller, J., Nelson, S., Samango-Sprouse, C. A., Spence, S., State, M., Tanzi, R. E., Coon, H., Dawson, G., Devlin, B., Estes, A., Flodman, P., Klet, L., McMahon, W. M., Minshew, N., Munson, J., Kovatska, E., Rodier, P. M., Schellenberg, G. D., Smith, M., Spence, M. A., Stodgell, C., Tepper, P. G., Wijsman, E. M., Yu, C. E., Roge, B., Mantoulan, C., Wittmeyer, K., Poustka, A., Felder, B., Klauck, S. M., Schuster, C., Poustka, F., Bolte, S., Feines- Matthews, S., Herbrecht, E., Schrotzer, G., Tsiantis, J., Papanikolaou, K., Maestriini, E., Bacchelli, E., Blasi, F., Carone, S., Toma, C., Van Engeland, H., de Jonge, M., Kemner, C., Koop, F., Langemeijer, M., Hjimans, C., Staal, W. G., Baird, G., Bolton, P. F., Rutter, M. L., Weisblatt, E., Green, J., Aldred, C., Wilkinson, J. A., Pickles, A., Le Couteur, A., Berney, T., McConachie, H., Bailey, A. J., Francis, K., Honeyman, G., Hutchinson, A., Parr, J. R., Wallace, S., Monaco, A. P., Barnby, G., Kobayashi, K., Lamb, J. A., Sousa, I., Sykes, N., Cook, E. H., Guter, S. J., Leventhal, B. L., Salt, J., Lord, C., Corsello, C., Hus, V., Weeks, D. E., Volkmar, F., Tauber, M., Fombonne, E., and Shih, A. (2007) Nat Genet 39, 319-328
20. Varoqueaux, F., Aramuni, G., Rawson, R. L., Mohrmann, R., Misssler, M., Gottmann, K., Zhang, W., Sudhof, T. C., and Brose, N. (2006) Neuron 51, 741-754
21. Chubykin, A. A., Atasoy, D., Etherton, M. R., Brose, N., Kavalali, E. T., Gibson, J. R., and Sudhof, T. C. (2007) Neuron 54, 919-931
22. Prange, O., Wong, T. P., Gerrow, K., Wang, Y. T., and El-Husseini, A. (2004) Proc Natl Acad Sci USA 101, 13915-13920
23. Chih, B., Engelma, H., and Scheiffele, P. (2005) Science 307, 1324-1328
24. Levinson, J. N., and El-Husseini, A. (2005) Neuron 48, 171-174
25. Futai, K., Kim, M. J., Hashikawa, T., Scheiffele, P., Shen, M., and Hayashi, Y. (2007) Nat Neurosci 10, 186-195
26. Conroy, W. G., Nai, Q., Ross, B., Naughton, G., and Berg, D. K. (2007) Dev Biol 307, 79-91
27. Misssler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R. E., Gottmann, K., and Sudhof, T. C. (2003) Nature 424, 939-948.
28. Zhang, W., Rohlmann, A., Sargysyan, V., Aramuni, G., Hammer, R. E., Sudhof, T. C., and Misssler, M. (2005) J Neurosci 25, 4330-4342
29. Sons, M. S., Busche, N., Strenzke, N., Moser, T., Ernsberger, U., Mooren, F. C., Zhang, W., Ahmad, M., Steffens, H., Schomburg, E. D., Plomp, J. J., and Misssler, M. (2006) Neuroscience 138, 433-446
30. Dudanova, I., Sedej, S., Ahmad, M., Masius, H., Sargsyan, V., Zhang, W., Riedel, D., Angenstein, F., Schild, D., Rupnik, M., and Missler, M. (2006) *J Neurosci* **26**, 10599-10613.
31. Hata, Y., Davletov, B., Petrenko, A. G., Jahn, R., and Sudhof, T. C. (1993) *Neuron* **10**, 307-315.
32. Hata, Y., Butz, S., and Sudhof, T. C. (1996) *J Neurosci* **16**, 2488-2494.
33. Biederer, T., and Sudhof, T. C. (2000) *J Biol Chem* **275**, 39803-39806.
34. Grootjans, J. J., Reekmans, G., Ceulemans, H., and David, G. (2000) *J Biol Chem* **275**, 19933-19941.
35. Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Sudhof, T. C. (2001) *J Cell Biol* **154**, 435-445.
36. Missler, M., Hammer, R. E., and Sudhof, T. C. (1998) *J Biol Chem* **273**, 34716-34723.
37. Goslin, K., Asmussen, H., and Banker, G. (1998) in *Culturing Nerve Cells* (Banker, G., and Goslin, K., eds), 2nd Ed., pp. 339-370, MIT Press, Cambridge.
38. Kaech, S., and Banker, G. (2006) *Nat Protoc* **1**, 2406-2415.
39. Ushkaryov, Y. A., Petrenko, A. G., Geppert, M., and Sudhof, T. C. (1992) *Science* **257**, 50-56.
40. Brunig, I., Scotti, E., Sidler, C., and Fritschy, J. M. (2002) *J Comp Neurol* **443**, 43-55.
41. Bolliger, M. F., Frei, K., Winterhalter, K. H., and Gloor, S. M. (2001) *Biochem J* **356**, 581-588.
42. Essrich, C., Lorez, M., Benson, J. A., Fritschy, J.-M., and Luscher, B. (1998) *Nature Neurosci* **1**, 563-571.
43. Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., Jackson, M. F., Lambert, J. J., Rosahl, T. W., Wafford, K. A., MacDonald, J. F., and Orser, B. A. (2004) *Proc Natl Acad Sci USA* **101**, 3662-3667.
44. Knuesel, I., Mastrocola, M., Zuellig, R. A., Bornhauser, B., Schaub, M. C., and Fritschy, J. M. (1999) *Eur J Neurosci* **11**, 4457-4462.
45. Levi, S., Grady, R. M., Henry, M. D., Campbell, K. P., Sanes, J. R., and Craig, A. M. (2002) *J Neurosci* **22**, 4274-4285.
46. Ullrich, B., Ushkaryov, Y. A., and Sudhof, T. C. (1995) *Neuron* **14**, 497-507.
47. Patzke, H., and Ernsberger, U. (2000) *Mol Cell Neurosci* **15**, 561-572.
48. Zeng, Z., Sharpe, C. R., Simons, J. P., and Gorecki, D. C. (2006) *Int J Dev Biol* **50**, 39-46.
49. Ichtchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Sudhof, T. C. (1995) *Cell* **81**, 435-443.
50. Tyzio, R., Represa, A., Jorquera, I., Ben-Ari, Y., Gozlan, H., and Anksztejn, L. (1999) *J Neurosci* **19**, 10372-10382.
51. Ben-Ari, Y., Khalilov, I., Represa, A., and Gozlan, H. (2004) *Trends Neurosci* **27**, 422-427.
52. Ushkaryov, Y. A., and Sudhof, T. C. (1993) *Proc Natl Acad Sci USA* **90**, 6410-6414.
53. Dudanova, I., Tabuchi, K., Rohlmann, A., Sudhof, T. C., and Missler, M. (2007) *J Comp Neurol* **502**, 261-274.
54. Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamion, R. A., Hoshi, T., and Campbell, K. P. (2002) *Nature* **418**, 422-425.
55. Sara, Y., Biederer, T., Atasoy, D., Chubykin, A., Mozhayeva, M. G., Sudhof, T. C., and Kavalali, E. T. (2005) *J Neurosci* **25**, 260-270.
56. Dalva, M. B., Takasu, M. A., Lin, M. Z., Shamah, S. M., Hu, L., Gale, N. W., and Greenberg, M. E. (2000) *Cell* **103**, 945-956.
57. Kayser, M. S., McClelland, A. C., Hughes, E. G., and Dalva, M. B. (2006) *J Neurosci* **26**, 12152-12164.
58. Kim, S., Burette, A., Chung, H. S., Kwon, S. K., Woo, J., Lee, H. W., Kim, K., Kim, H., Weinberg, R. J., and Kim, E. (2006) *Nat Neurosci* **9**, 1294-1301.
59. Zeng, X., Sun, M., Liu, L., Chen, F., Wei, L., and Xie, W. (2007) *FEBS Lett* **581**, 2509-2516.
60. Li, J., Ashley, J., Budnik, V., and Bhat, M. A. (2007) *Neuron* **55**, 741-755.
FIGURE LEGENDS

FIG. 1. α-Neurexins induce clustering of the inhibitory synaptic scaffolding protein gephyrin but not the excitatory synaptic scaffolding protein PSD-95. A. COS cells expressing the neurexin variants tagged intracellularly with CFP were overlaid on hippocampal neurons pre-grown for 8-10 days in culture. After one day of coculture, cells were fixed and immunolabeled for PSD-95, gephyrin, and synapsin; overlays are shown of PSD-95 (green) plus synapsin (blue), or gephyrin (red) plus synapsin (blue). Phase contrast images (top row) show the positions of transfected COS cell nuclei (C), neuronal processes, and occasional neuronal cell bodies (N). Endogenous inter-neuronal synapses have apposed clusters of postsynaptic PSD-95 or gephyrin and presynaptic synapsin, thus clusters appear turquoise or purple, respectively, in the color overlays (arrows). In contrast, clusters of gephyrin lacking associated synapsin appear red in the color overlay and were induced by all α-neurexins (arrowheads). By alignment with the phase contrast images, induced clusters of gephyrin can be seen corresponding to sites of neuronal process contact with the neurexin-expressing COS cells, and were frequently but not always localized near the edges of transfected COS cells, presumably where the COS cells come into closest contact with the neighboring neurons. Clusters of PSD-95 lacking associated synapsin were not induced by any α-neurexins. B. In sister coculture experiments, control COS cells expressing membrane-associated CFP (mCFP) did not induce clustering of either PSD-95 or gephyrin. All neurexin constructs used in this paper contain the splice site 4 insert. See also Fig. S1 for single channel larger field of view and phase contrast images corresponding to the cropped field shown here for neurexin2α-CFP. Scale bar, 10 µm.

FIG. 2. β-Neurexins induce clustering of the inhibitory synaptic scaffolding protein gephyrin and the excitatory synaptic scaffolding protein PSD-95. COS cells expressing the neurexin variants tagged intracellularly with CFP were overlaid on hippocampal neurons pre-grown for 8-10 days in culture. After one day of coculture, cells were fixed and immunolabeled for PSD-95, gephyrin, and synapsin; full field overlays are shown of PSD-95 (green) plus synapsin (blue), or gephyrin (red) plus synapsin (blue). Endogenous inter-neuronal synapses have apposed clusters of postsynaptic PSD-95 or gephyrin and presynaptic synapsin, thus clusters appear turquoise or purple, respectively, in the color overlays (arrows). In contrast, clusters of PSD-95 or gephyrin lacking associated synapsin appear green or red, respectively, in the color overlays and were induced by all β-neurexins (arrowheads). By alignment with the phase contrast images, induced clusters of PSD-95 and gephyrin can be seen corresponding to sites of neuronal process contact with the neurexin-expressing COS cells, and were frequently but not always localized near the edges of transfected COS cells, presumably where the COS cells come into closest contact with the neighboring neurons. While the induced clusters of gephyrin and PSD-95 appear to overlap at low resolution, at higher resolution they usually resolve into separate clusters side by side (enlarged regions at bottom of triple overlay of PSD-95 green, gephyrin red, and synapsin blue). All neurexin constructs used in this paper contain the splice site 4 insert. See also Fig. S2 for single channel larger field of view and phase contrast images corresponding to the cropped fields shown here for neurexin2β-CFP. Scale bar, 10 µm.

FIG. 3. Quantitation of induced clustering of gephyrin but not PSD-95 by α-neurexins. Quantitation from random transfected COS cells was performed to assess the total integrated intensity of associated PSD-95 or gephyrin clusters that did not overlap with synapsin; values were normalized to the integrated intensity associated with COS cells expressing membrane-targeted CFP in sister cultures (grey lines at 1). This quantitation confirmed significant clustering of gephyrin by all neurexin variants tested, but of PSD-95 only for the β-neurexin constructs and not the α-neurexins (** p < 0.005, * p < 0.05 compared with mCFP by t-test; n = 32-35 cells per construct combined from 2 independent cultures).

FIG. 4. α-Neurexins induce clustering of neurolig 2 but not neurolig 1/3/4. A. COS cells expressing the neurexin variants tagged intracellularly with CFP (blue) were overlaid on hippocampal neurons pre-grown for 10-12 days in culture. After one day of coculture, cells were fixed and immunolabeled for neurolig 1/3/4 (with an antibody that recognizes all three) or neurolig 2 (red) and synapsin (green). Whereas the β-neurexin constructs induced clustering of neurolig 1/3/4 and neurolig 2 in contacting dendrites (red or pink not associated with green), the α-neurexins induced clustering of
neuroligin 2 but not neuroligin 1/3/4. Scale bar, 10 µm. B. Cocultures were scanned and the COS cells with the strongest apparent associated clusters were imaged and quantitated. The total integrated intensity of associated neuroligin that did not overlap with synapsin was normalized to a value of 100 for neurexin 1β. Significant differences among constructs were found (p<0.001 ANOVA; n = 4-11 cells per construct).

FIG. 5. α-Neurexins induce clustering of GABA<sub>A</sub> receptor γ2 subunit. A COS cell expressing neurexin-2α-CFP (blue) is shown here in contact with two major hippocampal neuron dendrites. Clustering of the GABA<sub>A</sub> receptor γ2 subunit (red) in the absence of glutamic acid decarboxylase (GAD; green) is prominent where the dendrites contact the neurexin-2α-CFP-expressing COS cell (pink). In contrast, the endogenous synaptic clusters of GABARγ2 at axon-dendrite contacts are associated with GAD-positive GABAergic input (yellow). Similar induced clustering of GABARγ2 in COS cell-neuron coculture was observed for the other α and β neurexin constructs (not shown). Scale bar, 10 µm.

FIG. 6. Postsynaptic protein clustering by neurexin-1α requires LNS6 and is negatively modulated by upstream sequences. A. Neurexin 1β, 1α, and derivatives of 1α mutated in a predicted calcium-binding residue in LNS6 (1αD1176A), or lacking module A (1αBC), or lacking modules A and B (1αC) were tagged intracellularly with CFP (blue) and tested for postsynaptic protein clustering activity in the COS cell-neuron coculture assay. Activity was considered positive if the contacting dendrites exhibited any clusters of PSD-95, gephyrin, or neuroligin 2 (red) lacking synapsin (green). Neurexin 1αD1176A had no detectable clustering activity. For PSD-95, all neurexin-1α derivatives also had no detectable clustering activity, PSD-95 clustering was only observed in response to neurexin 1β (compare these images with Figures 1, 2 and 4). For gephyrin and neuroligin 2, the clustering activity of neurexin 1αC was indistinguishable from that of 1α. In contrast, the clustering activity of neurexin 1αC was higher than that of 1α and intermediate between that of 1α and 1β. Scale bar, 10 µm. B. Quantitation of the percentage of expressing COS cells exhibiting non-synaptic clusters in contacting dendrites confirmed these differences in relative synaptogenic activity (p<0.001 by ANOVA; * p <0.001 by t-test compared with neurexin 1α; n = 4 cultures with 10-78 cells scored each).

FIG. 7. Expression of neurexin splice variants lacking the S4 insert increases with development in hippocampus and cortex. The relative levels of neurexin variants containing (+) or lacking (-) inserts at splice site 4 was determined by RT-PCR from rat hippocampus and cortex at embryonic day 18 (E18), postnatal day 11 (P11) and adult (Ad). A. Representative agarose gel electrophoresis of RT-PCR products. B. Quantitative analysis revealed a significant increase in the percentage of each variant lacking the S4 insert between E18 and P11. Statistical analyses represent differences between P11 and other groups as determined by ANOVA with Tukey’s post hoc test; *p<0.05, † p<0.005; n=3.

FIG. 8. Alternative splicing at the S4 site is regulated by NMDA receptor activity in hippocampal cultures. Neurons from E18 rat hippocampus were cultured in the chronic presence (+) or absence (-) of the NMDA receptor antagonist APV. Cultures were harvested at the indicated ages and RT-PCR performed to assess the relative levels of neurexin variants containing or lacking inserts at splice site 4. A. Representative agarase gel electrophoresis of RT-PCR products. G, GAPDH loading control. DIV, days in vitro. B. Quantitative analysis revealed a significant increase in the percentage of neurexin 2β and decrease in the percentage of neurexin 3α and 3β lacking the S4 insert with chronic NMDA receptor blockade. Statistical analyses represent differences between untreated cells and age-matched APV-treated cells as determined by ANOVA with Tukey’s post hoc test; * p<0.05, † p<0.005; n=6. Semi-quantitative RT-PCR in comparison with GAPDH did not reveal any significant differences in total expression level of neurexin 1α, 1β, 2α, 2β, 3α or 3β (+ and - S4 forms combined) between control cells or cells chronically treated with APV (data not shown).

SUPPLEMENTAL FIGURE LEGENDS

FIG. S1. Neurexin 2α induces clustering of the inhibitory synaptic scaffolding protein gephyrin but not the excitatory synaptic scaffolding protein PSD-95. Larger field of view single channel and
additional overlay images are shown to more clearly illustrate the results of Fig. 1 (with synapsin image duplicated to aid visual alignment of clusters in columns). COS cells (C in phase contrast image, upper left) expressing neurexin 2α tagged intracellularly with CFP were overlaid on hippocampal neurons (N in phase contrast image, upper left) pre-grown for 8-10 days in culture. After one day of coculture, cells were fixed and immunolabeled for PSD-95, gephyrin, and synapsin. Endogenous inter-neuronal synapses have apposed clusters of postsynaptic PSD-95 or gephyrin and presynaptic synapsin (arrows), thus clusters appear turquoise or purple, respectively, in the color overlays. In contrast, clusters of gephyrin lacking associated synapsin (arrowheads) appear red in the color overlay and were observed specifically at contact sites with COS cells expressing neurexin 2α. Clusters of PSD-95 lacking associated synapsin were not induced by neurexin 2α. Scale bar, 10 µm.

FIG. S2. Neurexin 2β induces clustering of the inhibitory synaptic scaffolding protein gephyrin and the excitatory synaptic scaffolding protein PSD-95. Larger field of view single channel and additional overlay images are shown to more clearly illustrate the results of Fig. 1 (with synapsin image duplicated to aid visual alignment of clusters in columns). COS cells (C in phase contrast image, upper left) expressing neurexin 2α tagged intracellularly with CFP were overlaid on hippocampal neurons (N in phase contrast image, upper left) pre-grown for 8-10 days in culture. After one day of coculture, cells were fixed and immunolabeled for PSD-95, gephyrin, and synapsin. Endogenous inter-neuronal synapses have apposed clusters of postsynaptic PSD-95 or gephyrin and presynaptic synapsin (arrows), thus clusters appear turquoise or purple, respectively, in the color overlays. In contrast, clusters of PSD-95 or gephyrin lacking associated synapsin (arrowheads) appear green or red, respectively, in the color overlays and were observed specifically at contact sites with COS cells expressing neurexin 2β. While the induced clusters of PSD-95 and gephyrin appear to overlap at low resolution, at higher resolution they typically resolve into separate clusters side by side (see enlarged regions in Fig. 2). Scale bar, 10 µm.

FIG. S3. α-Neurexins did not induce clustering of GABA_A receptor α5 subunit. COS cells expressing neurexin 2α tagged intracellularly with CFP were overlaid on hippocampal neurons pre-grown for 16-18 days in culture. After one day of coculture, cells were incubated live with antibodies against either the α5 or the γ2 GABA_A receptor subunit. After washing, cocultures were fixed, permeabilized, and incubated with anti-synapsin and then secondary antibodies. Neurexin 2α induced surface clusters of GABA_A receptor γ2 lacking apposed synapsin (arrowheads, red in color overlay), which tended to be of greater intensity than the endogenous GABA_A receptor γ2 clusters apposed to synapsin (arrows, yellow in color overlay). In contrast, neurexin 2α did not induce detectable clustering of GABA_A receptor α5. As previously described, endogenous GABA_A receptor α5 was distributed more diffusely along dendrites compared with γ2. Some inhomogeneity in α5 immunoreactivity was observed along many dendrites, but the inhomogeneity was not more pronounced at contacts with COS cells expressing neurexin 2α. Scale bar, 10 µm.

FIG. S4. Confocal imaging supports surface association of the neurexin deletion constructs. Each indicated neurexin construct tagged intracellularly with CFP was expressed in HEK cells. A z series through one cell is shown for neurexin 1β (upper left). For comparison, single optical sections are shown through the middle of expressing HEK cells for each construct. In each case, association of fluorescence with the cell periphery and not interior structures suggests relative equal efficiency of surface localization. Scale bar, 10 µm.
Fig. 2
Fig. 3
Fig. 6
Fig. 7
Fig. 8
Fig. S3

$\text{GABAR}_\alpha 5$ $\text{GABAR}_\gamma 2$

$\text{Neurexin}_2\alpha$-CFP $\text{Neurexin}_2\alpha$-CFP

$\text{GABAR}_\alpha 5$ $\text{GABAR}_\gamma 2$

Synapsin Synapsin

$\text{GABAR}_\alpha 5$ $\text{GABAR}_\gamma 2$

Synapsin Synapsin
Fig. S4
Induction of gabaergic postsynaptic differentiation by α-neurexins
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