Presynaptic Targeting of α4β2 Nicotinic Acetylcholine Receptors Is Regulated by Neurexin-1β

The mechanisms involved in the targeting of neuronal nicotinic acetylcholine receptors (AChRs), critical for their functional organization at neuronal synapses, are not well understood. We have identified a novel functional association between α4β2 AChRs and the presynaptic cell adhesion molecule, neurexin-1β. In non-neuronal tsA 201 cells, recombinant neurexin-1β and mature α4β2 AChRs form complexes. α4β2 AChRs and neurexin-1β also coimmunoprecipitate from rat brain lysates. When exogenous α4β2 AChRs and neurexin-1β are coexpressed in hippocampal neurons, they are robustly targeted to hemi-synapses formed between these neurons and cocultured tsA 201 cells expressing neuroligin-1, a postsynaptic binding partner of neurexin-1β. The extent of synaptic targeting is significantly reduced in similar experiments using a mutant neurexin-1β lacking the extracellular domain. Additionally, when α4β2 AChRs, α7 AChRs, and neurexin-1β are coexpressed in the same neuron, only the α4β2 AChR colocalizes with neurexin-1β at presynaptic terminals. Collectively, these data suggest that neurexin-1β targets α4β2 AChRs to presynaptic terminals, which mature by trans-synaptic interactions between neurexins and neuroligins. Interestingly, human neurexin-1 gene dysfunctions have been implicated in nicotine dependence and in autism spectrum disorders. Our results provide novel insights as to possible mechanisms by which dysfunctional neurexins, through downstream effects on α4β2 AChRs, may contribute to the etiology of these neurological disorders.

The clustering of ion channels or receptors and precise targeting to pre- and postsynaptic specializations in neurons is critical to efficiently regulate synaptic transmission. Within the central nervous system, neuronal nicotinic acetylcholine receptors (AChRs) regulate the release of neurotransmitters at presynaptic sites and mediate fast synaptic transmission at postsynaptic sites of neurons (2). These receptors are part of a family of acetylcholine-gated ion channels that are assembled from various combinations of α2–α10 and β2–β4 subunits (3). AChRs participate in the regulation of locomotion, affect, reward, analgesia, anxiety, learning, and attention (4, 5).

The α4β2 subtype is the most abundant AChR receptor expressed in the brain. Multiple lines of evidence support a major role for α4β2 AChRs in nicotine addiction. α4β2 AChRs show high affinity for nicotine (6) and are located on the dopaminergic projections of ventral tegmental area neurons to the medium spiny neurons of the nucleus accumbens (7, 8). Furthermore, β2 AChR subunit knockout mice lose their sensitivity to nicotine in passive avoidance tasks (9) and show attenuated self-administration of nicotine (10). α4 AChR subunit knockout mice also exhibit a loss of tonic control of striatal basal dopamine release (11). Finally, experiments with knock-in mice expressing α4β2 AChRs hypersensitive to nicotine demonstrate that α4β2 AChRs indeed mediate the essential features of nicotine addiction including reward, tolerance, and sensitization (12). High resolution ultrastructural studies show that α4 subunit-containing AChRs are clustered at dopaminergic axonal terminals (13), and a sequence motif has been identified within the α4 AChR subunit cytoplasmic domain that is essential for receptor trafficking to axons (14). However, the mechanisms underlying the targeting and clustering of α4β2 AChRs to presynaptic sites in neurons remain elusive.

Recently, bi-directional interactions between neurexins and neuroligins have been shown to promote synapse assembly and maturation by fostering pre- and postsynaptic differentiation (reviewed in Refs. 15–17). The neurexins are encoded by three genes corresponding to neurexins I–III (18, 19), each encoding longer α-neurexins and shorter β-neurexins, because of differential promoter use. Neurexins recruit N- and P/Q-type calcium channels via scaffolding proteins, including calmodulin.

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5 The abbreviations used are: AChR, neuronal nicotinic acetylcholine receptor; Ab, antibody; ASD, autism spectrum disorders; BAC, baculovirus bacmid; GABA, γ-aminobutyric acid; NLG, neuroligin-1; NRX, neurexin-1β lacking the insert at splice site 4; mAb, monoclonal antibody; HA, hemagglutinin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DIV, day(s) in vitro; miRNA, micro-RNA interference-expressing construct; VSV-G, vesicular stomatitis virus G.
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associated serine/threonine kinase (20), to active zones of presynaptic terminals (21, 22). Recently, α-neurexins were shown to specifically induce GABAergic postsynaptic differentiation (23). Neurexins, postsynaptic binding partners of neurexins, cluster N-methyl-D-aspartate receptors and GABA receptors by recruiting the scaffolding proteins PSD-95 (postsynaptic density 95) and gephyrin, respectively (24, 25). Interestingly, neurexins and neurlgins also modulate the postsynaptic clustering of α3-containing AChRs in chick ciliary ganglia (26, 27). In this study, using multiple experimental strategies, we provide evidence for the formation of complexes between neurexin-1β and α4β2 AChRs and a role for neurexin in the targeting of α4β2 AChRs to presynaptic terminals of neurons.

EXPERIMENTAL PROCEDURES

Generation of Constructs—All of the constructs were made by PCR using appropriate pairs of forward and reverse synthetic oligonucleotide primers (Invitrogen) and *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). Rat α4, rat β2, and chicken α7 AChR subunit cDNAs were cloned into the mammalian cell expression vector pEF6/Myc-His A as described previously (28). Mouse neurexin-1β lacking the insert at splice site 4 with an extracellular VSV-G epitope tag at the mature N terminus of the protein (NRX) and mouse neurlgin-1 with an extracellular HA epitope tag at the mature N terminus of the protein (NLG) were kind gifts from Dr. Peter Scheiffele (29). The reading frame of full-length mouse NRX (NRX1–447) was amplified by PCR and subcloned between the EcoRI and XbaI sites of an extracellular VSV-G epitope tag at the mature N terminus of the protein (NRX) and mouse neurlgin-1 with an extracellular HA epitope tag at the mature N terminus of the protein (NLG) were kind gifts from Dr. Peter Scheiffele (29). The reading frame of full-length mouse NRX (NRX1–447) was amplified by PCR and subcloned between the EcoRI and XbaI sites of pEF6A vector. Truncation mutants were also made by PCR to create NRXΔC (NRX1–389) lacking the C-terminal cytoplasmic domain and NRXΔEC (Δ47–360) lacking the entire extracellular domain. Numbering includes the VSV-G tag.

Antibodies—The following antibodies (Abs) were used: rat mAbs to the α4 AChR subunit (mAb 299) and to the β2 AChR subunit (mAbs 295 and 270); a mouse mAb to the α7 AChR subunit (mAb 306); a goat polyclonal Ab against the cellular domain. Numbering includes the VSV-G tag of full-length mouse NRX (NRX1–447); a goat polyclonal Ab against the mature N terminus of the protein (NLG) were kind gifts from Dr. Peter Scheiffele (29). The reading frame of full-length mouse NRX (NRX1–447) was amplified by PCR and subcloned between the EcoRI and XbaI sites of pEF6A vector. Truncation mutants were also made by PCR to create NRXΔC (NRX1–389) lacking the C-terminal cytoplasmic domain and NRXΔEC (Δ47–360) lacking the entire extracellular domain. Numbering includes the VSV-G tag.

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Neuroligins, postsynaptic binding partners of neurexins, cluster AChRs to presynaptic terminals of neurons. Recently, neuroligins were shown to specifically induce GABAergic postsynaptic differentiation (23). While neuroligins are associated with diverse receptor types, several lines of evidence support a novel role for neuroligins in neuronal differentiation. First, knockout and deletion studies have shown that knockdown of neuroligins during embryonic development leads to abnormalities in neuronal connectivity, including the formation of abnormal dendritic spines (24, 25). Second, neuroligins are expressed in developing neuronal cells, and their expression increases during neuronal differentiation (26, 27). Third, neuroligins are localized to active zones of AChRs (28, 29), suggesting a direct interaction between these two proteins. Finally, neuroligins are localized to presynaptic terminals (21, 22), where they are likely to function as scaffolding proteins, organizing the clustering of AChRs and other synaptic proteins (23).

In conclusion, while the role of neuroligins in neuronal differentiation remains to be fully elucidated, these studies suggest that neuroligins may play a crucial role in the development of neuronal circuits. Further investigations are needed to determine the precise mechanisms by which neuroligins regulate neuronal differentiation, and to understand their role in the development of neuronal circuits.
room temperature. The beads were washed with PBS several times and finally with 200 mM glycine once and then stored at 4 °C in PBS containing 0.1% sodium azide. Frozen rat brains were homogenized and solubilized in 1% Nonidet P-40 buffer as previously described (28). Detergent-solubilized brain extracts (typically 1–3 ml) were precleared with 50 μl of protein G-Sepharose bead slurry and then incubated with 50 μl of mAb covalently cross-linked protein G-Sepharose beads (~5–10 μg of antibody/50 μl of beads) for 3–4 h at 4 °C. The beads were washed and eluted with sample buffer (lacking β-mercaptoethanol to avoid reduction of the disulfide linkage of the IgG chains) at 60 °C for 30 min, and then β-mercaptoethanol was added to the eluted samples prior to analysis by SDS-PAGE.

**Immunoblotting**—Following separation using SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membrane and incubated with diluted Abs in PBS containing 5% nonfat milk powder. The binding of the primary Abs to proteins was detected using appropriate secondary Abs as previously described (30).

**Quantitation of Cell Surface α4β2 AChR and Neurexin**—Cell surface α4β2 AChRs and NRX were measured using an enzyme-linked immunoassay previously described (28). Briefly, transfected, tsA 201 cells (0.5 × 10⁶ cells/well) were blocked with 3% BSA/PBS and incubated for 1 h with anti-β2 subunit (mAb 295) or anti-VSV-G antibodies in 3% BSA/PBS, washed, fixed with formaldehyde (3%), washed, and blocked again. The cells were incubated with horseradish peroxidase-conjugated goat anti-rat secondary Ab for 1 h in the presence of 3% BSA, washed, and incubated with 300 μl of the horseradish peroxidase-substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) for 1 h. The absorbance of the supernatant was then measured at 655 nm in a Beckman spectrophotometer. The values obtained using this assay are the mean ± S.E. and were statistically analyzed using an analysis of variance test. The significance level was set at p < 0.05. The nonspecific background to nontransfected cells was typically <0.5% of the total binding observed for transfected cells.

**Immunostaining and Imaging**—For the mixed neuron/tsA 201 cell assays, the cultures were fixed in 4% paraformaldehyde, 4% sucrose, Hanks’ balanced salt solution (with Ca²⁺ and Mg²⁺), pH 7.3 (15 min at room temperature), blocked with 3% normal goat serum, 3% BSA, Hanks’ balanced salt solution with 0.2% Triton X-100 (30 min at room temperature), and incubated with the appropriate primary (overnight at 4 °C) and secondary (90 min at room temperature) antibodies. Coverslips were mounted onto slides with ProLong Gold antifade reagent (Invitrogen). The cells were visualized using an Olympus IX81 spinning disc confocal microscope (Tokyo, Japan) with a xenon arc illumination source through a 60× (numerical aperture, 1.42) or 40× (numerical aperture, 1.35) Olympus oil immersion objective. Single-plane fluorescence images were captured using a Hamamatsu EM camera, and the images were processed using the Slide Book version 4.2 software. When the observed fluorescence intensity of antibody staining observed was weak, post-acquisition intensities of images were adjusted in the different channels using the gamma function of the slide book software to enhance visibility of axons and terminal in the figures shown. In all cases, the essential features of the original images were not altered. The figures were then processed with Adobe Photoshop CS.

**Statistics**—Targeting quantification was determined from 29–71 cells/condition from three independent experiments. Random neuroligin-1-expressing cells were imaged, and the targeting of the constructs was quantified as the number of neurons with targeting number of neuroligin-1 cells contacted. The values obtained are the means ± S.E. and were statistically analyzed by a Student’s t test.

**RESULTS**

α4β2 AChRs in the central nervous system are targeted to presynaptic terminals, but the mechanisms underlying their recruitment remain unclear. We investigated the possibility that β-neurexins, which are also highly enriched at axon terminals, have a functional role in the synaptic targeting of α4β2 AChRs. A neurexin-1β isoform was tested in subsequent functional studies with recombinant α4β2 AChRs.

**Neurexin-1β Forms Complexes with Recombinant α4β2 AChRs in tsA 201 Cells**—To determine whether NRX forms complexes with recombinant α4β2 AChRs, we coexpressed VSV-G-tagged neurexin-1β (NRX) with the α4 and the N-terminal FLAG-tagged β2 AChR subunits (labeled as α4β2FLAG AChRs) by transfecting tsA 201 cells with their respective cDNAs. Forty-eight hours post-transfection, 1% Nonidet P-40-solubilized cell lysates were incubated with FLAG M2 antibody covalently attached to agarose beads. Proteins eluted from these beads were then fractionated by SDS-PAGE and subjected to immunoblot analyses using Abs recognizing the α4 AChR subunit, the β2 AChR subunit, and the VSV-G tag. NRX was found in complexes with Nonidet P-40-solubilized α4β2FLAG AChRs (Fig. 1A). To confirm that the complex formation between NRX and α4β2 AChRs is not an artifact of detergent solubilization, we mixed Nonidet P-40-solubilized extracts from cells expressing NRX alone and cells expressing α4β2FLAG AChRs alone in a pulldown experiment using FLAG M2 beads (Fig. 1B). No NRX was coimmunoprecipitated with α4β2FLAG AChRs, indicating that the complex formation between NRX and α4β2 AChRs was not induced by detergent solubilization but instead was due to complex formation within the cell membrane.

To further verify that NRX forms complexes with assembled α4β2 AChRs, tsA 201 cells were cotransfected with untagged α4β2 AChRs and NRX and processed as in Fig. 1A. However, in this case (Fig. 1C), the α4β2 AChRs and their associated proteins were captured with a ligand that has a high affinity for α4β2 AChRs (BAC-conjugated to Affi-Gel 401 resin) (35). When α4β2 AChRs and NRX were coexpressed, BAC-conjugated beads captured both α4 and β2 AChR subunits, as well as NRX (Fig. 1C, BAC affinity capture, first lane). As a control for BAC capture specificity, lysates were incubated with 10 μM nicotine for 15 min prior to the addition of the BAC-coupled beads. Pretreatment with nicotine blocked the binding of BAC to the receptor complex (Fig. 1C, BAC, second lane). Additionally, BAC failed to capture the α4 AChR subunit if it was not coexpressed with the β2 AChR subunit (data not shown), indicating that only fully formed pentamers are affinity-purified. When the N-terminal HA-tagged neuroligin-1 (NLG), a trans-
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**FIGURE 1. NRX forms complexes with α4β2 AChRs in vitro.** A, coimmunoprecipitation of NRX with recombinant α4β2 AChRs from tsA 201 cells. tsA 201 cells were transfected with VSV-G-tagged NRX and untagged or FLAG-tagged α4β2 AChRs, as indicated along the bottoms of the blots, and were then lysed and immunoprecipitated (IP) with FLAG M2 beads. Lysates (input) and immunoprecipitates were immunoblotted for α4 (mAb 299), NRX (rabbit polyclonal anti-VSV-G), and β2 (goat polyclonal anti-β2), indicated along the right side of the image. Detergent-solubilized extract from the cells coexpressing VSV-G-tagged NRX and α4β2FLAG AChRs was incubated with IgG-coupled beads as an additional control (IgG immunoprecipitates). B, NRX does not coimmunoprecipitate with α4β2FLAG AChRs when they are transfected separately, and the lysates are combined. Detergent-solubilized extracts from the tsA201 cells expressing α4β2FLAG AChRs alone and tsA 201 cells expressing VSV-G-tagged NRX alone were mixed and pulled down using M2 beads. The eluates were subjected to SDS-PAGE, followed by Western blotting with the same antibodies as in A. No band for NRX was observed in the IP lane. C, copurification of NRX with assembled and mature recombinant α4β2 AChRs from tsA 201 cells using Bac affinity purification. Protein complexes captured with Bac-conjugated beads show the presence of the α4, β2 subunits, and NRX (BAC capture, lane 1). Pretreatment with 10 μM nicotine blocked binding of BAC to the mature α4β2 AChRs complexes (BAC capture, lane 2). N-terminal HA-tagged neurexin-1, the trans-synaptic partner of NRX, was not detected (BAC capture, lane 3), suggesting that the complex formation between NRX and mature α4β2 AChRs is specific. A faint nonspecific band is detected in the cells lysates with the VSV-G antibody (B, α4β2FLAG input lane; C, α4β2 + NLG-HA input lane; empty vector-transfected lysates (data not shown)).

synaptic binding partner of NRX, was coexpressed with α4β2 AChRs and incubated with BAC, the anti-HA antibody did not detect NLG in the pulldown (Fig. 1C, BAC, third lane), suggesting that the complex formation between NRX and α4β2 AChRs is specific.

**Neurexin-1β Forms Complexes with Native α4β2 AChRs Isolated from Rat Brain**—To determine whether neurexin-1β forms complexes with native α4β2 AChRs, as it does with recombinant α4β2 AChRs, 1% Nonidet P-40-solubilized rat brain membrane extracts were incubated with a β2 AChR subunit-specific mAb (mAb 295 or 270) or nonspecific rat IgGs (as a control), and the eluates were fractionated by SDS-PAGE and immunoblotted using Abs to the α4 AChR subunit, the β2 AChR subunit, and neurexin-1 (that was reported to cross-react with both the 1α and 1β isoforms). Both α4 and β2 AChR subunits are captured by the anti-β2 antibody. Note that the endogeneous levels of α4β2 AChRs in the lysates lanes of the blot are below the threshold for detection by the anti-α4 and anti-β2 AChR antibodies (Fig. 2A). The anti-neurexin antibody (P-15) detects a ~66-kDa band in both the lysate lane and the β2 immunoprecipitation lane (Fig. 2A, IP, NRX). No band of this size was observed in the pulldown using the nonspecific IgG as a control. Furthermore, no bands corresponding to neurexin-1α isoforms expected at ~165 kDa were observed in either the lysates or the pulldowns (Fig. 2A). Hence, we were unable to experimentally determine whether other neurexin-1α isoforms also form complexes with the α4β2 AChRs. When the immunoblots were probed with an anti-neurulin1-1 Ab, a strong band of the expected size (~110 kDa) was detected in the lysate but was absent in the precipitate captured with the anti-β2 AChR antibody (Fig. 2B). Additionally, an antibody against N-cadherin, a protein expressed in both pre- and post-synaptic membranes, did not detect this protein in the pull-down. These data suggest that neurexin-1β and the α4β2 AChRs are present in specific complexes in vivo.

Because the evidence that neurexins form complexes with native α4β2 AChRs relied on the use of a commercially generated anti-neurexin goat polyclonal antiserum (P-15, sc-1334; Santa Cruz) that has not been extensively characterized by other investigators, we additionally verified that this antiserum could recognize recombinant NRX expressed in tsA 201 cells (Fig. 2C). Equal amounts of samples from two eluates of pull-down experiments, one from cells coexpressing α4β2FLAG AChRs and VSV-G-tagged NRX and another from cells coexpressing α4β2FLAG AChRs and VSV-G-tagged NRX lacking its C terminus (NRXΔC), were loaded in parallel, and two sets of blots were probed with neurexin I or VSV-G antiserum. The results show that the neurexin I antiserum recognized both NRX and NRXΔC and that this antiserum weakly binds full-length NRX but is specific in its binding. Stronger reactivity of the Abs with the NRXΔC compared with the full-length NRX is observed possibly because truncation of the C terminus in the NRXΔC construct facilitates increased Ab access to highly antigenic terminal residues of the peptide epitope originally used to raise this antiserum. It is possible that our inability to detect the neurexin-1α isoforms may also be due to conformational masking of this epitope by the extracellular domains of the neurexin-1α isoforms.

**Neurexin-1β Does Not Affect the Expression Levels of α4β2 AChRs**—To investigate the functional significance of the interaction of NRX with the α4β2 AChRs, we first determined whether it affected the steady state levels of recombinant α4 or β2 AChR subunits. Either the pEF6A vector (as a control) or NRX was coexpressed in tsA 201 cells with α4β2 AChRs, and 48 h after transfection, the cells were lysed, separated by SDS-PAGE, and subjected to immunoblot analyses using Abs to the α4 and β2 AChR subunits. No significant change in the steady state levels of the α4 or β2 AChR subunits was observed, sug-
suggesting that NRX does not play a role in the early events that regulate AChR subunit stability (Fig. 3A).

Next, we assessed whether coexpression of NRX with α4β2 AChRs altered the steady state levels of either the α4β2 AChRs or NRX itself on cell surface membranes. Surface expression of the α4β2 AChRs was measured using an Ab to the extracellular domain of the β2 AChR subunit (mAb 295) in conjunction with a previously described enzyme-linked immunoassay (28). Similarly, the surface expression level of the NRX was measured with this same assay but using an Ab to the VSV-G tag. The coexpression of α4β2 AChRs with NRX did not significantly change their surface expression levels compared with when they were expressed alone (Fig. 3, B and C). The results suggest that NRX does not affect the trafficking of α4β2 AChRs to the cell surface membrane and vice versa and thus their rates of exo- or endocytosis to cell surface membranes of tsA 201 cells.

Neurexin-1β Induces Presynaptic Targeting of α4β2 AChRs in Rat Hippocampal Neurons—To test whether the presynaptic maturation functions of neuromeric included recruitment of α4β2 AChRs to synaptic terminals, we transfected rat hippocampal neurons with α4 and β2 AChR subunits and NRX cDNAs and cocultured them with tsA 201 cells transfected with neuroligin–1-HA (NLG). A similar in vitro assay for neuromeric-neuroligin signaling has been used extensively by other investigators (33). Neurons expressing α4β2 AChRs, in the absence of exogenous NRX, exhibited a low level (~11.1 ± 7.4%, n = 71) of AChR accumulation at contact sites formed with tsA 201 cells expressing NLG (Fig. 4A). It is important to note that this low level accumulation was qualitatively very different from those observed when NRX was coexpressed in neurons. These boutons were quite small and frequently did not recruit NLG to the contact sites (Fig. 4A, arrowhead), suggesting they were most likely immature synaptic boutons.

In contrast, when α4β2 and NRX were coexpressed, the α4β2 AChRs targeted robustly to sites of contact between neurons and NLG-expressing tsA 201 cells (Fig. 4B, arrows). In addition, the contact sites were frequently enlarged and quite elaborated, consistent with a synapse maturation function described previously for neurexin-neuroligin interactions (33). NLG-expressing tsA 201 cells recruited exogenous NRX to contact sites in 96.1 ± 3.9% of the neurons coexpressing NRX and α4β2 AChRs and recruited exogenous α4β2 AChRs in 69.1 ± 18% of the cells examined (n = 56). When NRX targeting is set to 100%, α4β2 AChRs cotargeted with NRX 72.9 ± 18.5% of the time (n = 52). As a control for specificity of the staining and targeting, we expressed NRX with the β2 AChR subunit (which is not targeted to the cell surface without the α4 subunit and thus should not be targeted to contact sites) in neurons and

![Figure 2. Neurexin-1β forms complexes with α4β2 AChRs in vivo.](image)

**FIGURE 2. Neurexin-1β forms complexes with α4β2 AChRs in vivo.** A. coimmunoprecipitation of NRX and α4β2 AChRs from whole rat brain lysates. Rat brains were homogenized, solubilized in 1% Nonidet P-40 lysis buffer, and incubated with beads conjugated to rat IgGs or rat anti-β2 (mAb 295). Lysate and immunoprecipitates (IP) were immunoblotted with rat anti-α4 (mAb 299), goat polyclonal anti-neurexin-1 (P-15), or goat polyclonal anti-β2 (C-20) antibodies. β, neurolgin 1 does not coimmunoprecipitate with α4β2 AChRs. Rat brain lysate and immunoprecipitates were incubated with rat anti-β2 (mAb 270) and immunoblotted with rat anti-α4 (mAb 299) and a mouse monoclonal anti-neuroligin 1 antibody. C. neurexin I antiserum cross-reacts with recombinant neurexin-1β. Samples were from the two eluates of pulldown experiments, one from cells coexpressing α4β2 AChRs and VSV-G-tagged NRX and another from cells coexpressing α4β2FLAG AChRs and VSV-G-tagged NRXΔC. The same amounts of samples were loaded side by side, and two sets of blots were probed with neurexin I or VSV-G antisera. Neurexin I antiserum is also capable of recognizing the recombinant mouse VSV-G-tagged NRX and VSV-G-tagged NRXΔC.

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A

\[ \alpha 4 \beta 2 + \text{vector} \quad \alpha 4 \beta 2 + \text{NRX} \]

B

\[ \% \text{ Surface AChR Expression} \]

\[ \alpha 4 \beta 2 + \text{vector} \quad \alpha 4 \beta 2 + \text{NRX} \]

C

\[ \% \text{ Surface NRX Expression} \]

\[ \text{NRX} \quad \text{NRX+vector} \quad \text{NRX+} \alpha 4 \beta 2 \]

did not observe any staining for the \( \beta 2 \) subunit at contact sites with NLG-expressing tsA 201 cells (supplemental Fig. S1). When the NRX construct lacking its extracellular domain (NRXΔEC) was coexpressed with \( \alpha 4 \beta 2 \) AChRs, both the mutant neurexin and \( \alpha 4 \beta 2 \) AChRs were poorly targeted to contact sites with NLG-expressing tsA 201 cells (3.7 \( \pm \) 3.7\%, \( n = 29 \)) (Fig. 4C).

To determine whether the hemi-synapses that formed between NRX-expressing neurons and NLG-expressing tsA 201 cells could recruit presynaptic vesicle markers, cocultures were costained with Abs to the \( \beta 2 \) AChR subunit, VSV-G, and synapsin-1, a synaptic vesicle protein. Nearly all the contact sites at which \( \alpha 4 \beta 2 \) AChRs and NRX were enriched were also positive for synapsin-1, indicating that these were indeed mature presynaptic terminals (Fig. 5, A–D, arrows). Synapsin-1 staining was also observed at the small number of vestigial synapses formed (as shown in Fig. 4) between neurons expressing \( \alpha 4 \beta 2 \) AChR alone and NLG-expressing tsA 201 cells (Fig. 5, E–G). Similarly, synapsin-1 staining was also observed at synaptic contacts sites between neurons expressing NRX alone and NLG-expressing tsA 201 cells (Fig. 5, H–J).

Multiple AChR subtypes, including \( \alpha 7 \), are present at presynaptic terminals (36). To study the relative specificity of targeting of \( \alpha 4 \beta 2 \) AChRs versus \( \alpha 7 \) AChRs by NRX in the same neuron, we coexpressed NRX, \( \alpha 4 \beta 2 \), and \( \alpha 7 \) in hippocampal neurons cocultured with NLG-expressing tsA 201 cells (Fig. 6). The \( \alpha 4 \beta 2 \) AChR (red) was detected in the soma, axon, and dendrites, whereas the \( \alpha 7 \) AChR (green) was only detected in the soma and dendrites. In these cells, the \( \alpha 4 \beta 2 \) AChR, but not the \( \alpha 7 \) AChR, colocalized with NRX (blue) in the axon (Fig. 6, A2–D2) and at presynaptic terminals (Fig. 6, A1–D1). Collectively, the results from these experiments provide evidence that NRX significantly enhances the presynaptic targeting of \( \alpha 4 \beta 2 \) AChRs in neurons.

We further investigated whether endogenous neurexin-1\( \beta \) had a role in the development of the vestigial synaptic terminals observed at \( \sim \)11% of contact sites in cocultures of neurons expressing only \( \alpha 4 \beta 2 \) AChRs and NLG-expressing tsA 201 cells. We developed micro-RNA interference-expressing constructs (miRNAs) to silence the expression of neurexin-1\( \beta \) and tested their ability to specifically knock down expression of NRX in tsA 201 cells expressing transfected \( \alpha 4 \beta 2 \) AChRs. At least one miRNA was able to silence the expression of NRX, as compared with the negative control miRNA (supplemental Fig. S2A). However, when the miRNA constructs were coexpressed with \( \alpha 4 \beta 2 \) AChRs in neurons cocultured with tsA 201 cells expressing NLG, we did not observe significant changes in the size of the synaptic boutons formed or in the extent to which low level targeting of \( \alpha 4 \beta 2 \) AChRs occurs at these contact sites on tsA cells expressing NLG (supplemental Fig. S2B). There was a trend toward an increase in puncta, but it did not reach significance. Overall, the base-line targeting of \( \alpha 4 \beta 2 \) AChRs observed was not significantly altered. This lack of phenotype could be due to multiple compensatory mechanisms, including functional redundancies among other the neurexin isoforms or other presynaptic cell adhesion molecules or because endogenous neurexin-1\( \beta \) does not mediate the low level of synaptic targeting observed with \( \alpha 4 \beta 2 \) AChRs expressed alone.
DISCUSSION

There is significant experimental evidence that the formation or maturation of pre- and post-synaptic specializations in neurons occurs through trans-synaptic, bi-directional signaling interactions between neurexins and neuroligins (23, 37–42). In this paper, we provide functional evidence that neurexin-1/4 targets 2 AChRs to presynaptic terminals of rat hippocampal neurons in a well established in vitro synapse maturation assay (33). Recently, this coculture assay was used to screen for novel synaptogenic proteins whose functions were validated in vivo (43). Additionally, detergent-solubilized complexes of native 2 AChRs isolated from brain contain neurexin-1β, but not neuroligin-1, which is another abundant cell adhesion molecule in the brain, providing physiological evidence for the presence of neurexin-1β in 2 AChR complexes in vivo. Collectively, these results establish a novel functional role for neurexin-1β in the targeting of AChRs to presynaptic terminals of neurons.

Differential splicing of five canonical alternative splice sites in the -neurexin transcripts and two in the -neurexin transcripts increases the potential complexity of neurexins to more than a thousand different isoforms (18), which differ only in their extracellular domains, whereas the transmembrane domains and cytoplasmic C-terminal tails are conserved. The physiological functions of these isoforms are not fully understood, but one role is for differential heterophilic interactions with their known postsynaptic binding partners, the neuroligins, which themselves are encoded by five differentially spliced genes that encode multiple neuroligin isoforms (22, 44). Interestingly, a role for neurexin-1 and neuroligin-1 in the postsynaptic recruitment of 3-containing AChRs in chick ciliary ganglion has been reported (26, 27). Our findings complement these studies and support a significant role for neurexins and neuroligins in the targeting of AChRs to synapses, in addition to their known functions in the recruitment of glutamate and GABA receptors to synapses.

The expression of NRX promotes presynaptic targeting of 2 AChRs in most, but not all, cultured hippocampal neurons. In addition, we found that 7 does not cotarget to terminals with 2 AChRs and NRX when expressed in the same neurons. These results indicate that the targeting of AChRs to presynaptic terminals may require additional factors specific to certain subpopulations of neurons. These factors may include the expression of accessory AChR subunits, including α5, α6, and β3, calmodulin-associated serine/threonine kinase-like molecules, and post-translational processes that regulate complex formation between AChRs and neurexins. Recently, calmodulin-associated serine/threonine

![Image](image.jpg)

FIGURE 4. 2 AChRs target with NRX to presynaptic terminals in hippocampal neurons. Single plane images of neurons that were transfected at 8 DIV, coplated with tsA 201 cells at 12 DIV and fixed, permeabilized, and immunostained at 14 DIV. A, neurons expressing 2 AChRs were coplated with tsA 201 cells expressing NLG. 2 AChRs and NRX-expressing cultures exhibit enhanced targeting of 2 AChRs to synapses (arrows) compared with 2 AChRs alone (A, arrowhead). C, neurons expressing 2 AChRs and NRX lacking its extracellular domain (NRXΔEC) were coplated with tsA 201 cells expressing NLG. D, quantification of results shown in A–C. *, p < 0.05, expressed as the means ± S.E. and analyzed using Student’s t test. Antibody combinations: A–C, anti-2 AChR (mAb 295, red), anti-VSV-G (green), and anti-HA (blue) antibodies. Scale bar, 10 μm.
kinase was shown to phosphorylate neurexin-1 (45), so it is possible that neurexin-1 binds different proteins depending on its phosphorylation state. Future experiments are necessary to sort out the full repertoire of neurexin isoforms involved in the synaptic targeting of the different AChR subtypes.

Our finding that neurexin-1β is involved in the targeting of α4β2 AChRs may have significant implications for the role of neurexins in the etiology of different neurological diseases typically associated with pathophysiological functions of AChRs. In this regard, it is significant that a recent high density genome-wide association study for nicotine dependence-linked single nucleotide polymorphisms in the neurexin-1 gene to the development of nicotine dependence and thus smoking behavior (46), and this association was replicated in an independent study (47). The α4β2 AChRs play a significant role in mediating the essential features of nicotine addiction including reward, tolerance, and sensitization (13). Thus, changes in the expression level of neurexin-1β could be expected to affect functions mediated by α4β2 AChRs. Little is known about how the neurexin-1α and -1β splicing is regulated to generate the predicted hundreds of neurexin-1 isoforms. Hence, it is possible that a regulatory single nucleotide polymorphism, linked to nicotine dependence, in an intron of neurexin-1α could modulate neurexin-1β levels. Alternatively, a specific neurexin-1α isoform may also influence AChR functions. Future, more challenging studies analyzing whether any of the hundreds of neurexin-1α isoforms also perform similar targeting functions are necessary to elucidate the linkage between neurexin-1 gene variants, α4β2 AChR synaptic targeting, and nicotine dependence. Nevertheless, our results provide support for a possible mechanism by which changes in neurexin-1 function could contribute to nicotine dependence.

The interactions between neurexin-1β and α4β2 AChRs may also shed light on the association between deficits in AChR, neurexin and neuroligin functions, and autism spectrum disorders (ASD). β2-Containing AChRs have been shown to regulate executive and social behaviors in β2 AChR subunit knock-out mice, and some of these affected behaviors have been reported to resemble behavioral deficits characteristic of ASD (48). Additionally, postmortem analyses of autistic patient brains show an extremely significant reduction in the expression levels of α4β2 AChRs in the cerebellar cortex (49, 50) and the parietal cortex (51). In addition, multiple recent linkage analysis studies (52, 53) and an analysis of structural variants in the β-neurexin genes (54) implicate neurexin-1 dysfunctions in ASD. Our results complement these studies and suggest that some behavioral deficits characteristic of ASD are highly likely to be due to defects in α4β2 AChR-mediated functions caused by neurexin or neuroligin dysfunctions.
