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Neuroligin Expressed in Nonneuronal Cells Triggers Presynaptic Development in Contacting Axons

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Summary

Most neurons form synapses exclusively with other neurons, but little is known about the molecular mechanisms mediating synaptogenesis in the central nervous system. Using an in vitro system, we demonstrate that neuroligin-1 and -2, postsynaptically localized proteins, can trigger the de novo formation of presynaptic structure. Nonneuronal cells engineered to express neuroligins induce morphological and functional presynaptic differentiation in contacting axons. This activity can be inhibited by addition of a soluble version of β-neurexin, a receptor for neuroligin. Furthermore, addition of soluble β-neurexin to a coculture of defined pre- and postsynaptic CNS neurons inhibits synaptic vesicle clustering in axons contacting target neurons. Our results suggest that neuroligins are part of the machinery employed during the formation and remodeling of CNS synapses.

Introduction

The excitable cells of the nervous system are joined into a network by connections called synapses (Sherrington, 1906). This synaptic network develops as axons extend from presynaptic neurons and grow to reach their correct postsynaptic partners. Extending axons are guided along specific trajectories and by cues present in their immediate environment (Sanes and Yamagata, 1999). Within the target region, however, growth cones still have the task of identifying correct targets and initiating synaptogenesis with them. As opposed to the increasing number of molecules known to serve as guidance cues, much less is known about the molecules mediating postsynaptic partner recognition and the initiation of synaptogenesis.

The molecular machinery involved in synapse formation has been best studied at the vertebrate neuromuscular junction, where agrin secreted by motoneurons triggers postsynaptic development in the target muscle and subsequent synaptogenesis (Sanes and Lichtman, 1999). Evidence exists that laminin β2 (Porter et al., 1995), basic fibroblast growth factor (Dai and Peng, 1995), and agrin itself (Campagna et al., 1995) can induce presynaptic differentiation directly in motoneurons; however, it is unclear which of these proteins is a relevant inducer in vivo (reviewed in Sanes and Lichtman, 1999). Molecules mediating the initial steps of synapse formation in the central nervous system (CNS) are not known. While agrin is found at central synapses, mice lacking agrin do not show any defects in CNS synaptogenesis (Serpinskaya et al., 1999). Other proteins that have been suggested to mediate CNS synaptogenesis include cadherin family proteins (Fannon and Colman, 1996; Uchida et al., 1996; Kohmura et al., 1998) and neurexins (Ushkaryov et al., 1992).

Neurexins were originally identified as receptors for the spider venom α-latrotoxin, which triggers massive neurotransmitter release (Ushkaryov et al., 1992). Unfortunately, it has so far not been possible to localize neurexins in the mammalian CNS at the ultrastructural level, and their synaptic localization therefore remains to be demonstrated. Thousands of neurexin variants are generated through alternative splicing, and this molecular diversity has been hypothesized to generate specificity during synaptogenesis (Missler et al., 1998). More recently, other proteins with sequence similarity to neurexin-1, -2, and -3 have been identified and were shown to mediate interactions between axons and glia cells (reviewed in Bellen et al., 1998). Since these proteins only partially share the domain structure of neurexin-1, -2, and -3, it is unclear whether they are bona fide neurexins (Missler et al., 1998).

While the presynaptic localization of neurexins remains to be demonstrated, neuroligin-1, a ligand of a specific splice variant of β-neurexins, has been clearly shown to localize to the postsynaptic compartment at excitatory synapses (Song et al., 1999). So far, three neuroligin genes have been described in the rat, and the predicted products of these genes are 52% identical at the amino acid level (Ichtchenko et al., 1995, 1996). Structurally, neuroligins possess in their cytoplasmic domain a PDZ binding motif that mediates interactions with synaptic scaffolding proteins such as PSD-95 (Irie et al., 1997). The extracellular domain of neuroligins consists largely of a region homologous to acetylcholinesterases, but the amino acids important for catalysis in acetylcholinesterases are not conserved in neuroligins, which lack esterase activity (Ichtchenko et al., 1995). However, the role of neuroligins at the synapse and the functional consequences of their interaction with β-neurexins are not understood.

We have recently begun to address the molecular mechanisms mediating the initiation of neuron–neuron synaptogenesis using an in vitro system that reconstitutes synaptogenesis between separately purified, bona fide pre- and postsynaptic neurons (D. E. Emerling, S. Y. Choi, and T. S., submitted). By culturing explants of neonatal pontine tissue, "purified" presynaptic axons can be prepared (Baird et al., 1992) and then paired in vitro with a purified preparation of their in vivo targets, cerebellar granule cells (Hatten, 1985). Upon contact with granule cells, clustering of synaptic vesicles is observed within pontine axons. This vesicle clustering is a central feature of the initiation of synaptogenesis observed in vivo for pontine axons contacting granule cells in the developing cerebellum (Mason, 1986). Using this in vitro system, we show here that the expression of

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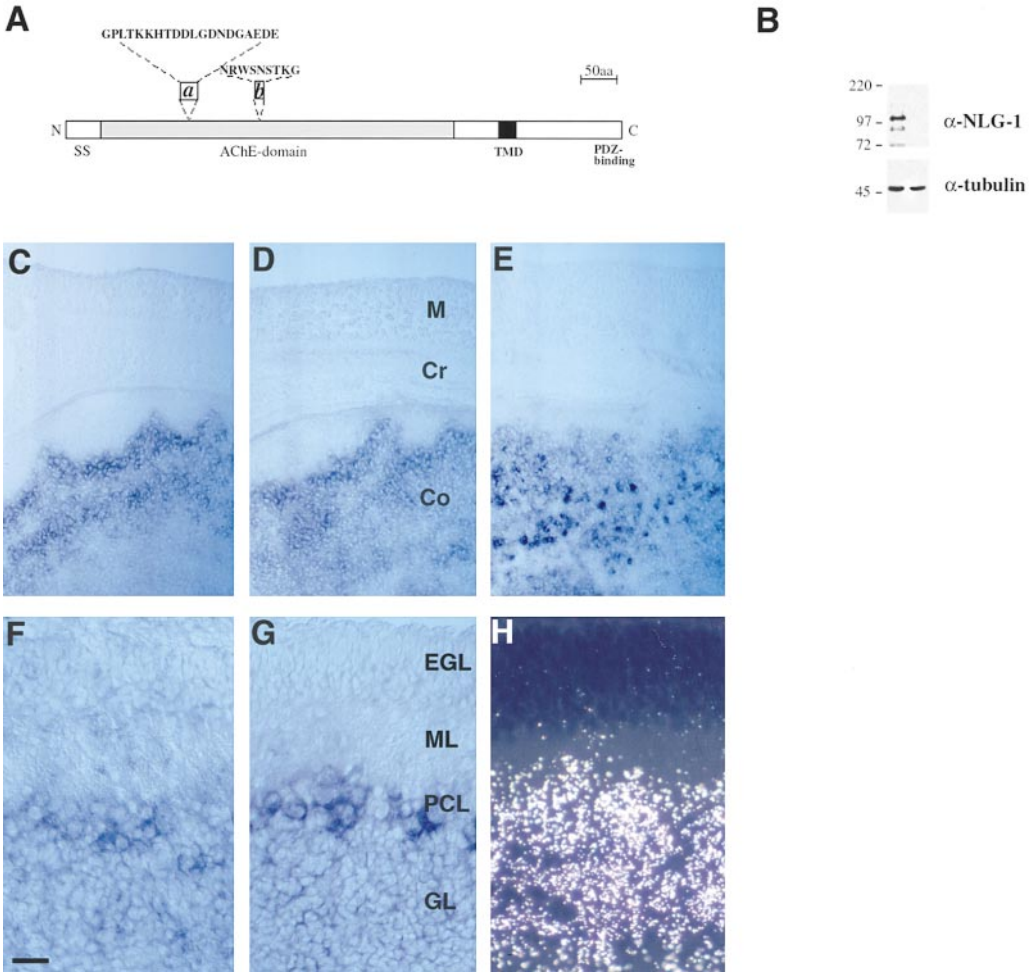

Figure 1. Neuroligins Are Expressed in the Cerebellum during Synaptogenesis

(A) Schematic drawing of mouse neuroligin-1 splice variants. Isolated cDNAs contained a novel insertion in the first alternatively spliced region (*a*). The second splice insertion (*b*) was identical to the previously described rat neuroligin-1 sequence. The amino acids of the splice insertions are given in single letter code. The signal sequence (SS) is marked, the acetylcholinesterase-homology (AChE) domain is shown in gray, and the predicted transmembrane domain (TMD) in black.

(B) Western blot using neuroligin-1 specific antibodies. Lysates of purified, cultured cerebellar granule cells or cultured astroglia were separated by SDS-PAGE and probed with affinity-purified anti-neuroligin-1 antibodies (upper panel) or anti-tubulin antibodies (lower panel). The position of molecular weight markers is indicated.

(C–H) Expression patterns of neuroligin-1 and neuroligin-2 in P2 heads and P8 cerebella analyzed with RNA in situ hybridization. Horizontal sections of P2 heads were hybridized with digoxigenin-labeled antisense probes specific for neuroligin-1 (C), neuroligin-2 (D), and GluR1 (E). Sagittal sections of P8 cerebella were hybridized with digoxigenin-labeled antisense probes specific for neuroligin-1 (F), neuroligin-2 (G), and a ^{35}S-labeled probe specific for neuroligin-1 (H). Digoxigenin-labeled probes were visualized with chromogenic reaction resulting in dark purple deposits in bright field (C–G). The ^{35}S-labeled probe was visualized by silver grains in darkfield illumination (H). M, muscle; Cr, cranium; Co, cortex; EGL, external germinal layer; ML, molecular layer; PCL, Purkinje cell layer; GL, granular layer.

The scale bar represents 100 μm for (C)–(E) and 25 μM for (F)–(H).

neuroligins by a nonneuronal cell is sufficient to induce presynaptic differentiation in axons that contact such cells. Our results suggest that neuroligins play an important role in the formation or remodeling of synapses.

Results

Neuroligin-1 and -2 Are Expressed during Synaptogenesis in the Cerebellum

To study the function of neuroligins in synaptogenesis in the cerebellum, we first isolated cDNAs encoding neuroligin-1 and -2 from mouse cerebellar granule cells (Figure 1). The previously characterized rat neuroligin-1 is subject to alternative splicing at two sites encoding portions of the extracellular domain (Ichtchenko et al., 1996). Sequencing of multiple cDNA clones revealed a similar heterogeneity of mouse neuroligin-1 isoforms. We found three different splice variants that carried two, one, or no insertions in the extracellular domain (Figure 1). While the overall predicted protein sequences of mouse neuroligin-1 and -2 were 98% identical to those of the rat homologs, surprisingly, in the splice insertion *a* of neuroligin-1, only one amino acid was identical to the sequence found in insertion *a* of the rat protein

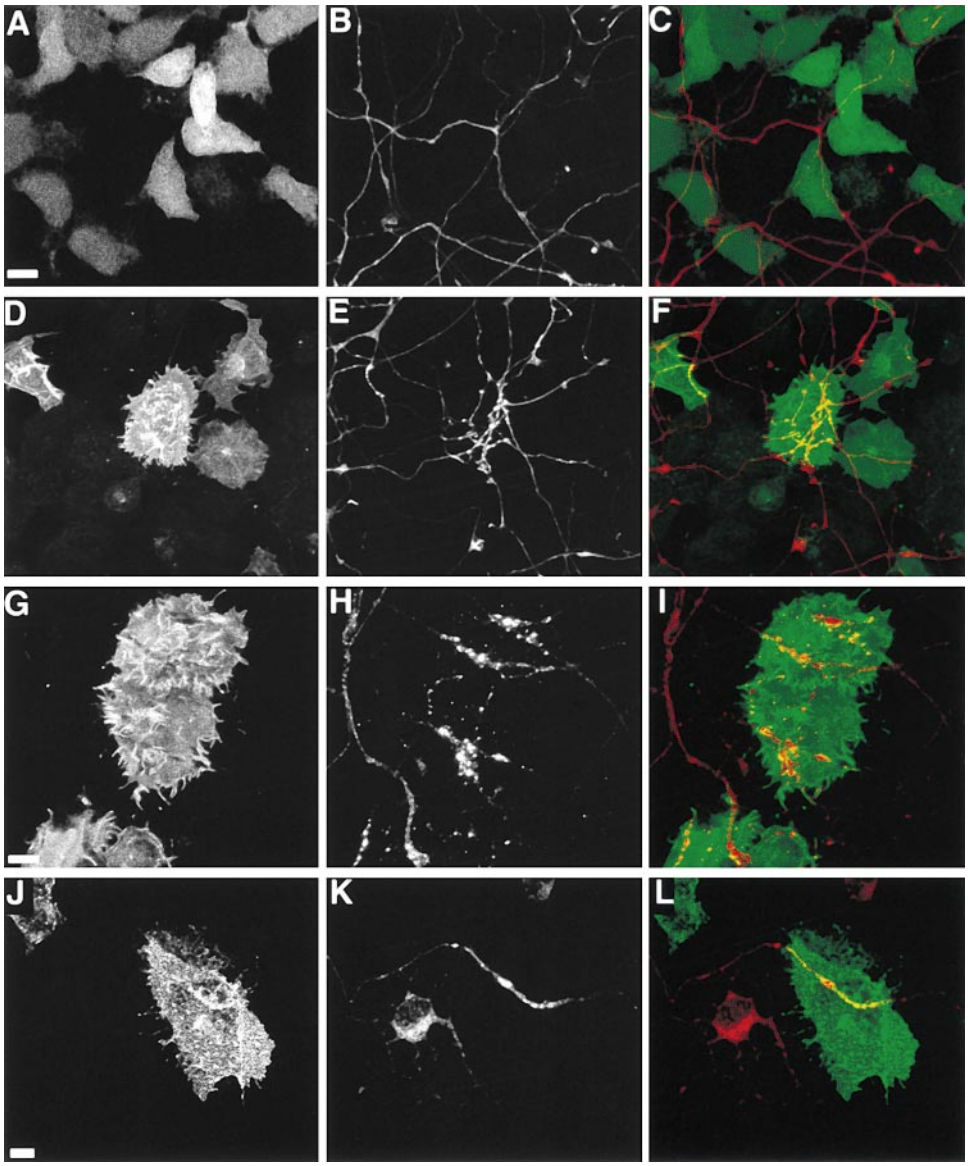

Figure 2. Neuroligin Expression in HEK293 Cells Causes Accumulation of Synapsin in Axons

HEK293 cells were transiently transfected with GFP (A–C), neuroligin-1*ab* (D–F and J–L), or neuroligin-2 (G–I) and were cocultured with pontine explants (A–I) or purified cerebellar granule cells (J–L). Fixed, immunostained cultures were analyzed by confocal microscopy. Fluorescence of GFP (A) or immunostaining with anti-HA antibodies to detect neuroligins (D, G, and J) is displayed in green in the overlay (C, F, I, and L). Immunostaining for synapsin in the pontine axons (B, E, H, and K) is displayed in red in the overlay (C, F, I, and L).
The scale bar in (A) represents 20 μm, 10 μm in (G), and 12 μm in (J).

(Figure 1A). Neuroligin expression in granule cells was confirmed by immunoblot analysis of cell lysates, but was not detected in lysates prepared from cultured cerebellar astrocytes (Figure 1B).

The expression pattern of neuroligin-1 and -2 in early postnatal development was analyzed by in situ hybridization of antisense RNA probes specific for neuroligin-1 or -2 on horizontal sections of P2 heads. As expected from previous Northern analysis (Ichtchenko et al., 1995, 1996), both neuroligins were found to be expressed exclusively in the brain with no detectable specific signal in other tissues such as muscle. The overall distribution was comparable to that obtained with a glutamate-receptor-specific antisense probe (representative details in Figures 1C–1E and data not shown). The expression patterns of neuroligin-1 and -2 were largely overlapping, indicating that both genes are coexpressed in all brain areas rather than being restricted to small specific subsets of cells (Figures 1C–1E and data not shown).

Sagittal sections of P8 cerebellum were examined at higher resolution (Figures 1F–1H). Hybridization with neuroligin-1 and -2 specific antisense probes was detected in Purkinje cells and in cells in the granular layer (GL). Sense RNA probes did not give a significant signal (data not shown). In the EGL, where the granule cell precursors are generated, only very little expression was detected. This was further confirmed by hybridization with radiolabeled antisense probes (Figure 1H). As we

detected hybridization for neuroligin-1 and -2 in cerebellar granule cells and Purkinje cells at P8, both cell types express neuroligins during the time when they are undergoing synaptogenesis.

Nonneuronal Cells Engineered to Express Neuroligins Induce Accumulation of Synapsin in Contacting Axons

When pontine axons and granule cells are cocultured in vitro, synaptic vesicles cluster in pontine axons at points of contact with granule cells, as delineated by the accumulation of synapsin I, a peripheral membrane protein of synaptic vesicles (D. E. Emerling, S. Y. Choi, and T. S., submitted). No such synapsin concentration is observed when pontine axons contact nonneuronal cells such as GFP-expressing HEK293 cells (Figures 2A–2C). In contrast, when HEK293 cells were transfected with a construct encoding epitope-tagged neuroligin-1*ab* or neuroligin-2, dramatic accumulations of synapsin were observed (Figures 2D–2I). Synapsin became concentrated within single axons in very brightly staining structures of a variety of sizes at sites of contact with neuroligin-expressing cells. Axons containing synapsin accumulations were detected on 70%–80% of all transfected cells. These structures developed within the first 24 hr of coculture with pontine explants, a time-course comparable to that observed when granule cells are contacted. Synaptotagmin and synaptophysin, integral membrane proteins of synaptic vesicles, displayed similar accumulations at contact sites with neuroligin-1*ab*-expressing cells, whereas they remained distributed throughout the axons in small clusters if axons contacted GFP-expressing HEK293 cells (data not shown).

Accumulation of synaptic vesicle markers in response to neuroligin-expressing cells is not unique to pontine axons. Cerebellar granule cells, the in vivo target of pontine axons, also concentrated synapsin in single axons contacting neuroligin-1*ab*-expressing HEK293 cells (Figures 2J–2L). Therefore, one neuroligin splice variant confers activity for the axons of at least two different CNS neuronal types.

Conversely, the ability of a neuroligin-expressing, nonneuronal cell to induce the accumulation of synapsin in pontine axons was not limited to the HEK293 epithelial-derived cell line. COS-7 cells (fibroblasts) or, remarkably, cultured primary astrocytes (glial cells) transfected with the neuroligin-1*ab* expression construct also induced the formation of synapsin accumulations in axons (data not shown). In all subsequent experiments, we used HEK293 cells, as they are a better growth substrate for pontine axons compared to COS-7 cells and yielded the highest transfection efficiencies.

Synaptic Vesicles Undergo Depolarization-Dependent Turnover at Sites of Neuroligin-Induced Clustering

One salient feature of synaptic function is the regulated exocytosis of vesicles in response to depolarizaton of the presynaptic membrane, followed by a reinternalization of synaptic vesicle membrane. In order to determine whether the neuroligin-induced synaptic vesicle clusters could undergo regulated exocytosis, cocultures of transfected HEK293 cells and pontine explants were briefly (5 min) incubated in a depolarizing solution with an antibody raised against the luminal domain of synaptotagmin (Figures 3A–3E). Captured synaptotagmin antibodies, representing synaptic vesicles that had undergone exocytosis, were visualized with secondary antibodies (Figure 3B), while the distribution of the total synaptotagmin pool was revealed by an antibody directed against the cytoplasmic domain of the protein (Figure 3C). Synaptic vesicles localized on neuroligin-expressing cells clearly displayed exocytosis (Figure 3D). Importantly, large synaptotagmin-positive axonal structures not in contact with neuroligin-expressing cells showed only little vesicle turnover (Figure 3D, arrows). This indicates that the neuroligin-induced synaptic vesicle clusters are functionally distinct from these other accumulations (such as those found in growth cones). No detectable uptake was observed for a control immunoglobulin when applied under identical conditions (data not shown).

The vesicle turnover we observed was depolarization dependent (Figure 3E). The ratio of staining intensities obtained with the luminal and the cytoplasmic synaptotagmin antibodies was 0.76 ± 0.03 ($n = 20$) under depolarizing conditions and 0.25 ± 0.02 ($n = 13$) under standard culture conditions, indicating a 3-fold increase due to depolarization. This is comparable to what has been described for synaptic vesicles clustered at synapses in hippocampal cultures (Kraszewski et al., 1995).

Finally, we analyzed the distribution of CASK/Lin-2, a member of the MAGUK family of proteins, which localizes to synapses (Hsueh et al., 1998). CASK/Lin-2 binds to neurexin and is part of a complex that has been proposed to be involved in synaptic vesicle exocytosis (Butz et al., 1998). Both pontine axons and HEK293 cells were immunoreactive for CASK/Lin-2, with lower levels found in HEK293 cells than in axons (data not shown). Using triple immunostaining and serial *z*-sectioning in a confocal microscope, CASK/Lin-2 was found colocalized with the presynaptic vesicle clusters in pontine axons (Figures 3F–3I and see Experimental Procedures). Thus, not only synaptic vesicles, but also a potential structural scaffolding component is recruited upon axonal contact with neuroligin-expressing cells.

Neuroligin-Expressing Cells Induce Morphological Presynaptic Differentiation

We used electron microscopy to analyze the morphology of the induced vesicle clusters on the ultrastructural level (Figure 4). Pontine explants and HEK293 cells transfected with a construct encoding HA-tagged neuroligin-1*ab* were cocultured and then labeled at 37°C with anti-HA-tag primary and gold-conjugated secondary antibodies in order to identify transfected cells unambiguously.

Axonal processes were identified by their morphology and the typical pattern of microtubule bundles in cross section. Axons growing over untransfected HEK293 cells mostly displayed a rounded morphology in cross section and did not form extensive contacts with the cells (Figure 4B). Axonal contacts with neuroligin-expressing cells displayed a strikingly different morphology (Figure 4A). The axons and cells formed large, flattened areas of contact with closely apposed membranes. At several sites, electron dense material was

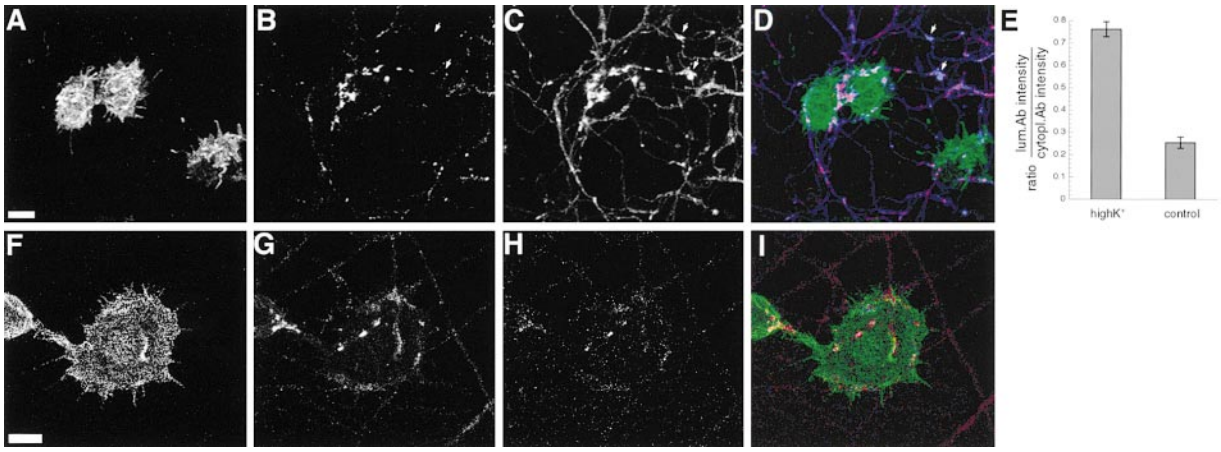

Figure 3. Synaptic Vesicles Undergo Turnover at Contacts Formed with Neuroligin-Transfected HEK293 Cells

HEK293 cells were transiently transfected with HA-tagged neuroligin-1*ab* and cocultured with pontine explants as described.

(A–E) Vesicle turnover in the cocultures was assayed as uptake of an antibody against the luminal domain of synaptotagmin in depolarizing buffer for 5 min (see Experimental Procedures). Bound synaptotagmin antibodies were visualized by secondary antibodies (B). The distribution of HA-tagged neuroligin-1*ab* was visualized by immunostaining using anti-HA antibodies (A), and the total pool of synaptotagmin was detected with a monoclonal antibody directed against the cytoplasmic domain of the molecule (C). In the overlay (D), HA-neuroligin-1*ab* is shown in green, bound synaptotagmin luminal domain antibodies in red, and total synaptotagmin in blue. Arrows mark structures strongly labeled with cytoplasmic synaptotagmin antibodies, but which display low luminal-domain-specific antibody uptake. (E) Turnover was quantified as described in Experimental Procedures for depolarization with high potassium (high K^+) and for nondepolarizing conditions (control).

The scale bar in (A) corresponds to 20 μm.

(F–I) Cultures were stained with rat anti-HA (F), rabbit anti-synapsin (G), and mouse anti-CASK antibodies (H). In the overlay (I), HA-neuroligin is shown in green, synapsin in red, and CASK in blue. Confocal settings were chosen such that most of the signal derived from CASK/Lin-2 in the HEK293 cells was not visualized.

The scale bar in (F) represents 15 μm.

observed at and between the contacting plasma membranes of the axon and the HEK293 cells. Most importantly, concentrations of vesicles were found at the membrane at contact sites (Figure 4A). The average size of the vesicles was 37.4 ± 0.4 nm (n = 123), a typical size for synaptic vesicles. Serial sections revealed individual vesicle clusters (46 ± 6 vesicles per cluster, n = 5) at multiple sites within a single axon–HEK293 interface (Figures 4C–4G). Such presynaptic morphologies were found only in processes in direct contact with neuroligin-expressing cells, while axons unable to contact the cell directly did not display these structures (Figure 4H). Therefore, neuroligin-expressing HEK293 cells induce contact-mediated presynaptic differentiation in axons.

Neuroligin Splice Variants Have Similar Activities In Vitro

Because we could detect several neuroligin isoforms by RT-PCR in cerebellar granule cells, we determined whether they were active in our coculture assay. Both neuroligin-1 and neuroligin-2, and all neuroligin-1 variants showed similar activities (Figure 5A). Across all conditions, 95% ± 4% of all measured synapsin immunostaining above a defined threshold (see Experimental Procedures) was found directly associated with neuroligin-expressing cells.

Expression of Other Adhesion Proteins Is Not Sufficient to Trigger Synaptic Vesicle Clustering

The effects we observed in axons in response to contact with neuroligin-expressing cells could be a consequence of overexpressing a neuronal cell adhesion molecule in nonneuronal cells. In order to address this issue, several other cell adhesion proteins were assayed for their ability to mimic the effects of neuroligin.

We tested the following proteins with adhesive properties in our coculture assay: N-cadherin, ephrinB1, TAG-1, agrin, and L1. N-cadherin is a homophilic cell adhesion molecule that localizes to mature synapses formed by pontine mossy fibers in vivo (Fannon and Colman, 1996). B-ephrins, such as ephrinB1, contain C-terminal PDZ binding motifs, cause cell aggregation in *trans* via their binding to EphB receptors, and localize to synapses (Torres et al., 1998). Agrin, a secreted protein of 200 kDa, accumulates at the cell surface when expressed in nonneuronal cells and might directly induce presynaptic differentiation in motoneurons (Campagna et al., 1995). In addition, we tested two immunoglobulin superfamily members, TAG-1 and L1, which are expressed in granule cells (Powell et al., 1997). Importantly, receptors for N-cadherin, ephrinB1, and L1 are found on pontine axons in vitro (data not shown).

When expressed on the cell surface of HEK293 cells, none of these proteins induced significant concentrations of synapsin in pontine axons upon contact (Figures 5A–5M). Induction of presynaptic differentiation in CNS axons is therefore a response inherent so far only to neuroligin-1 and -2.

Vesicle Clustering Is Mediated by Specific Sequences in the Esterase-Homology Domain of Neuroligin

To gain insight into the mechanism of neuroligin activity, we performed a structure-function analysis. The role of the intracellular domain was addressed with two cytoplasmic truncation mutants, NLGΔC1 and NLGΔC2,

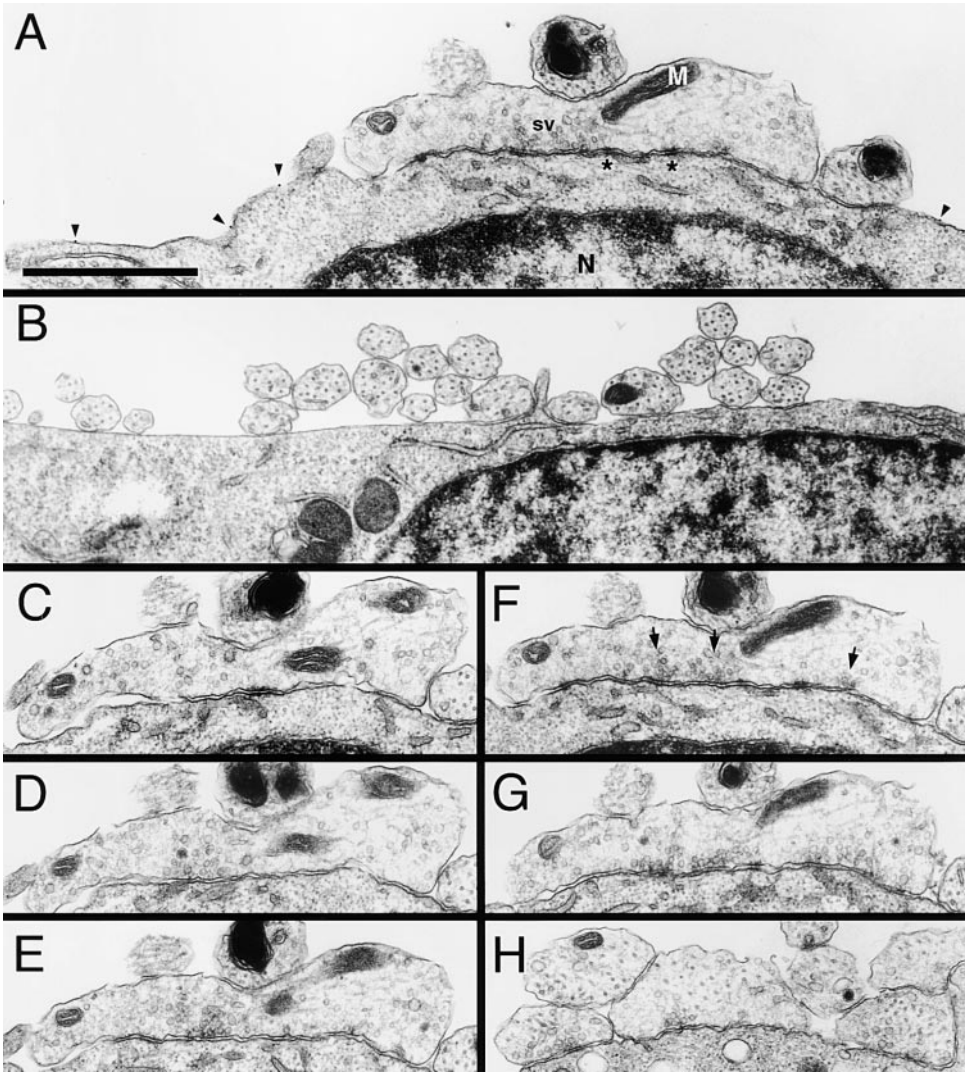

Figure 4. Neuroligin-Expressing Cells Induce the Formation of Presynaptic Structures

(A) Contact between pontine axons and a HEK293 cell transfected with an HA-tagged neuroligin-1*ab* expression construct. On the HEK293 cell surface, 15 nm gold particles (used to label transfected cells) are marked by arrowheads. Two junctional areas with electron dense material (asterisk, [*]), synaptic vesicle profiles (sv), a mitochondrion in the axon (M), and the nucleus of the HEK293 cell (N) are labeled.

(B) Similar view of a contact between pontine axons and mock-transfected HEK293 cells.

(C–G) Serial sections (0.1 μm thickness) of the contact in (A), showing the spatial extent and multiple sites of vesicle accumulation in a single axon-HEK293 contact. In panel (F), three individual vesicle clusters are marked by arrows.

(H) Several axonal processes growing over a single neuroligin-expressing HEK293 cell.

The scale bar is 1 μm.

lacking the C-terminal 40 and 72 amino acids of neuroligin, respectively (see Figure 6A). Since a mutant protein lacking the entire cytoplasmic domain was not transported to the cell surface of transfected cells, a chimeric protein was constructed carrying extracellular and transmembrane sequences of neuroligin fused to the cytoplasmic domain of CD8.

The extracellular portion of neuroligin consists of two separate domains: a stalk region with eight predicted O-glycosylation sites and a region homologous to acetylcholinesterase (35% identical, 53% similar residues). The importance of these domains was tested by two deletion mutants, NLGΔAChE and NLGΔO-glyc (see Figure 6A). All described deletion mutants and the CD8 chimera were transported efficiently to the cell surface of HEK293 cells, as confirmed by immunofluorescence staining and biochemical assays (see Figure 6C and data not shown). None of the cytoplasmic deletion mutants showed a significant difference compared to the wild-type protein in the ability to induce vesicle clustering in pontine axons when transfected into HEK293 cells (Figure 6B). Deletion of the potentially O-glycosylated stalk region in the extracellular domain had a very minor effect on clustering activity, whereas deletion of the acetylcholinesterase-homologous domain reduced activity to background levels (Figure 6B).

To test whether specific sequences within the esterase domain are required for the clustering activity, we replaced either the entire domain or the last 184 amino acids of the domain with the homologous sequences

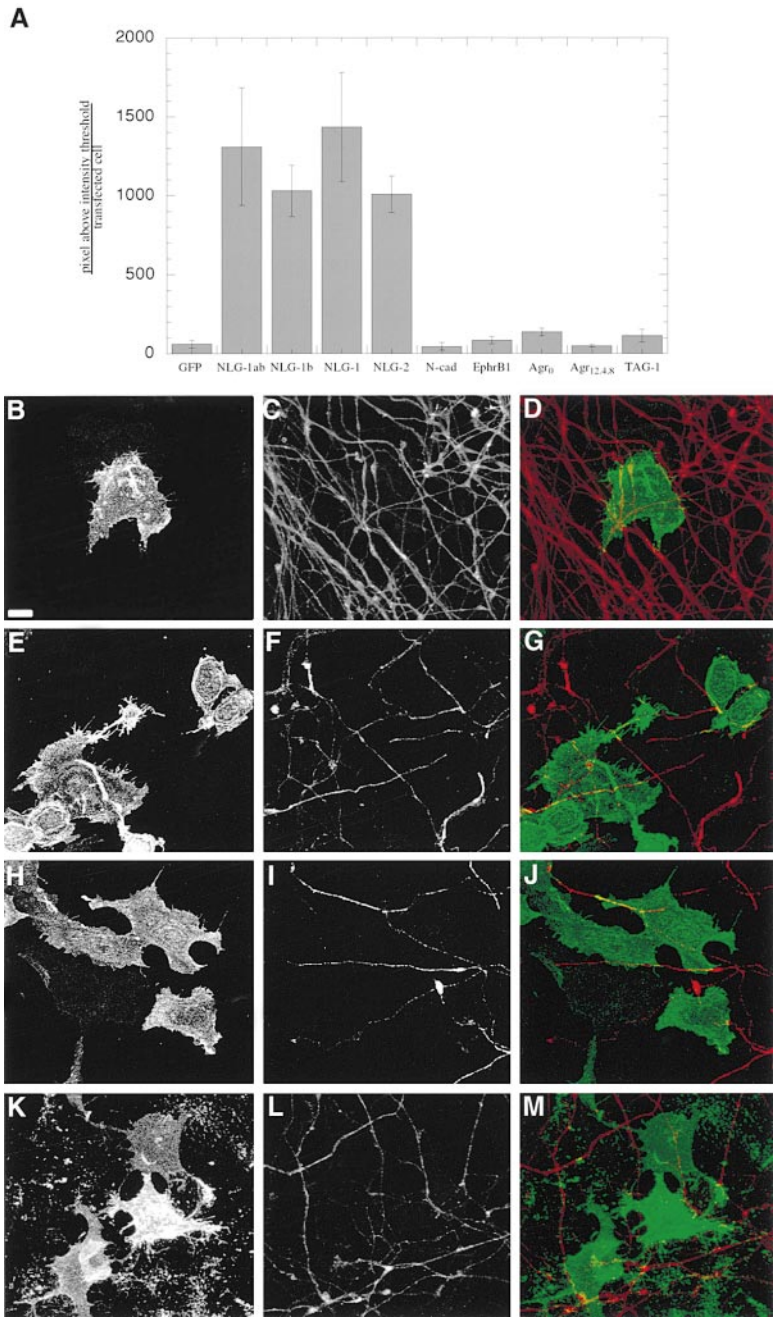

Figure 5. Overexpression of Adhesion Molecules Is Not Sufficient to Induce Synaptic Vesicle Clustering in Pontine Axons

(A) HEK293 cells were transfected with constructs encoding GFP, neuroligin-1 having splice insertions *a* and *b* (NLG-1*ab*), only insertion *b* (NLG-1*b*), no insertion (NLG-1), neuroligin-2 (NLG-2), N-cadherin, ephrinB1, agrin0, agrin12.4.8, or TAG-1. For each transfected protein, 12 randomly taken images were quantified. The mean (with standard error) is shown (see Experimental Procedures). (B–J) Immunostaining for ephrinB1 (B), N-cadherin (E), TAG-1 (H), agrin0 (K), and synapsin (C, F, I, and L) is shown. Immunostaining for each of the transfected molecules is displayed in green in the overlay images (D, G, J, and M), synapsin in red. Since some of the agrin is secreted from the HEK293 cells, some deposits of the protein are observed on the culture substratum (K).

The scale bar in (A) represents 20 μm.

from mouse acetylcholinesterase (NLGAChEswap, and NLGswap184, see Figure 6A). Both mutants were efficiently transported to the cell surface of transfected HEK293 cells. Furthermore, NLGAChEswap exhibited specific esterase activity of >2000 units/mg, demonstrating that the chimeric protein was correctly folded. However, both proteins completely lacked vesicle clustering activity when expressed in HEK293 cells and cocultured with pontine explants (Figure 6B).

Finally, we addressed the role of the transmembrane domain. We took advantage of a natural GPI-anchored splice variant of mouse acetylcholinesterase that is expressed in hematopoietic cells (Li et al., 1991). Like the chimera NLGAChEswap, this GPI-anchored acetylcholinesterase (AChE-GPI) lacked activity in our coculture assay. However, when the esterase domain was replaced with the corresponding sequences of neuroligin, the resulting GPI-anchored protein NLG-GPI induced vesicle clustering in pontine axons (Figure 6B). Therefore, specific sequences in the acetylcholinesterase domain of neuroligin are necessary and sufficient when expressed in HEK293 cells to induce presynaptic differentiation in axons.

Neuroligin Activity Is Inhibited by Soluble β-Neurexin

Neuroligins were originally identified biochemically as ligands of β-neurexins (Ichtchenko et al., 1995). To examine whether the activity of neuroligin in our coculture assay might be mediated by its ability to interact with β-neurexin in the axon, we tested all neuroligin mutants

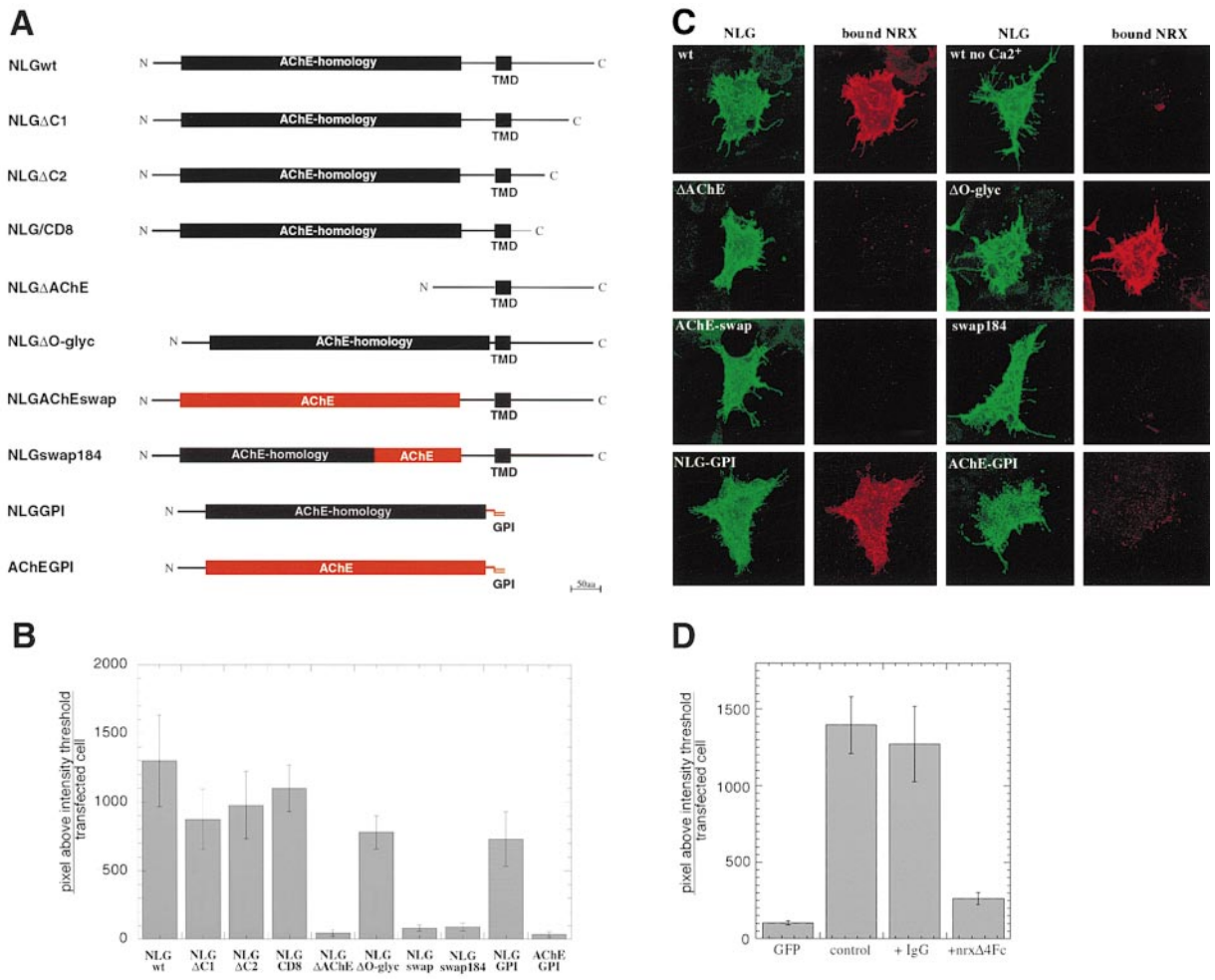

Figure 6. Vesicle Clustering Activity of Neuroligin Requires Specific Sequences in the AChE-Homology Domain and Is Blocked by Soluble Neurexin

(A) Schematic representation of neuroligin-1*ab* mutants. NLG wt is neuroligin-1*ab* wild type; NLGΔC1 and NLGΔC2 carry deletions in the cytoplasmic tail; in NLG/CD8, the entire cytoplasmic tail of neuroligin was replaced by the cytoplasmic tail of CD8; in NLGΔAChE, the AChE-homologous domain was deleted; in NLGΔO-glyc, the stalk region with all predicted O-glycosylation sites was removed; in NLGAChEswap and NLGswap184, either the entire AChE-homologous domain or only the C-terminal 184 amino acids of neuroligin were replaced by the corresponding sequences of mouse AChE; AChEGPI is the glycosylphosphatidylinositol-anchored form of mouse AChE; in NLGGPI, the esterase sequences of AChEGPI were replaced by the homologous region of neuroligin-1*ab*. Sequences derived from neuroligin are shown in black, sequences from mouse AChE in red, and sequences from CD8 in green. TMD is transmembrane domain.

(B) Quantitation of synapsin accumulation in pontine axons induced by various neuroligin mutants. Pontine explants and transfected HEK293 cells were cocultured and the distribution of synapsin quantified as described above.

(C) Neurexin binding of neuroligin mutants. HEK293 cells transfected with mutant cDNAs were incubated with neurexin-1β lacking splice insertion 4. To complex free calcium (wt no Ca^{2+}), 10 mM EGTA was added to the incubation buffer. Immunostaining for neuroligin (green) and bound neurexin (red) is shown. Confocal images of representative examples were recorded with identical settings for laser power, gain, and offset.

(D) HEK293 cells transfected with GFP or neuroligin-1*ab* were cocultured with pontine explants under standard conditions (GFP and control), in presence of 50 μg/ml control IgG (+IgG), or in presence of 50 μg/ml neurexin-Fc fusion protein lacking splice insertion 4 (+nrxΔ4Fc). The distribution of synapsin was quantified as described above.

for their β-neurexin binding activity (Figure 6C). No binding was observed on untransfected HEK293 cells; however, cells expressing HA-tagged, neuroligin-1 bound β-neurexin in a calcium-dependent manner. Binding of β-neurexin to cells transfected with neuroligin expression constructs strikingly paralleled the activity of the mutant proteins in the functional coculture assay (Figure 6C).

To test whether the activity of neuroligin could be inhibited by binding neurexin, we added soluble β-neurexin to a coculture of neuroligin-expressing HEK293 cells and pontine explants. Since β-neurexin produced in *E. coli* bound to neuroligin only with relatively low affinity, we constructed a neurexin-IgG fusion protein (nrxΔ4-Fc) that was purified from transfected HEK293 cells. Addition of nrxΔ4-Fc to the coculture at a concentration of 50 μg/ml reduced neuroligin-induced vesicle clustering in axons by 80%, whereas a control IgG had

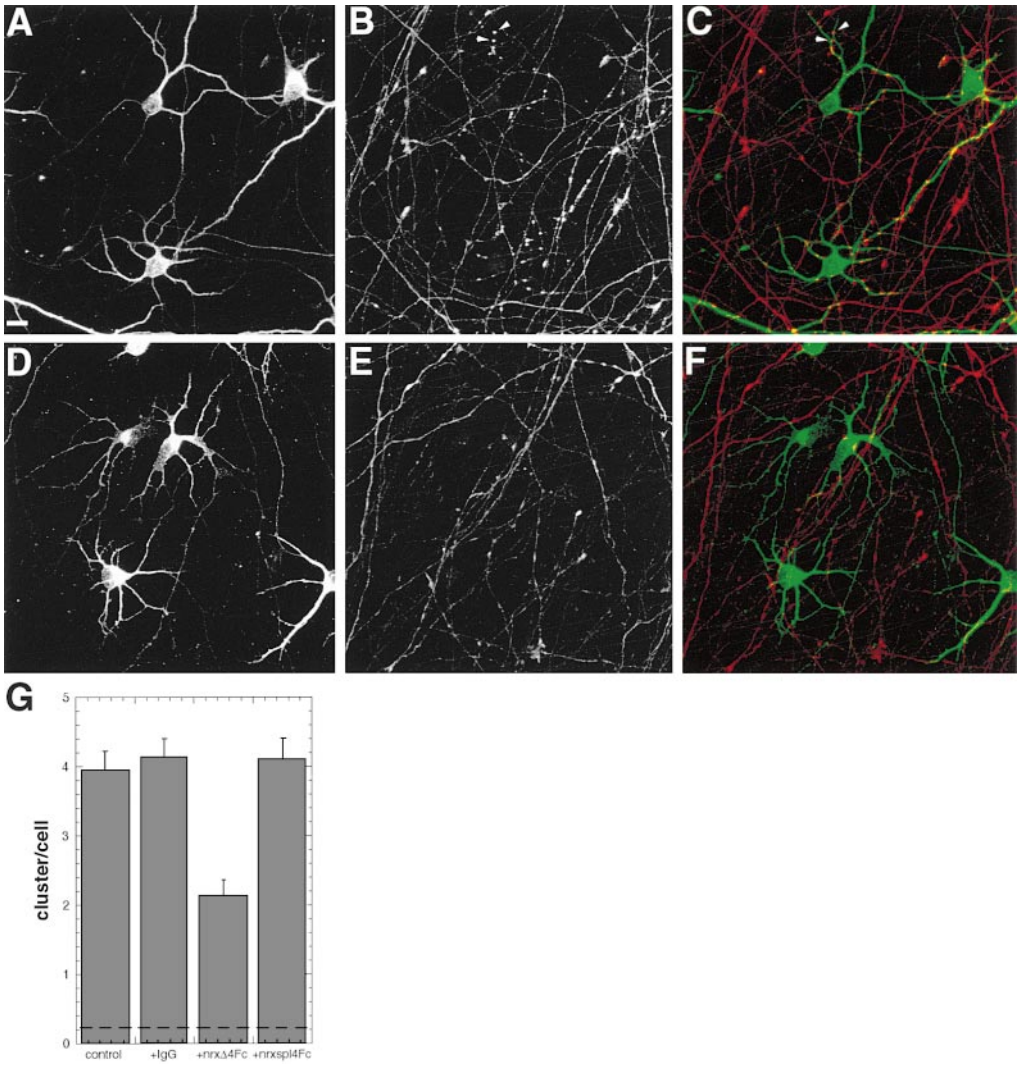

Figure 7. Soluble β-Neurexin Reduces Granule Cell–Induced Clustering of Synapsin in Pontine Axons

Purified cerebellar granule cells isolated from synapsin KO animals were cocultured with pontine explants in the presence of 50 μg/ml control IgG (A–C) or 50 μg/ml nrxΔ4-Fc (D–F). Cultures were stained with antibodies anti-MAP2 (A, D, green in overlays C and F) and anti-synapsin (B and E, red in overlay). In (B) and (C), two examples of synapsin clusters are marked by arrowheads. Bright synapsin clusters $\geq 1\ \mu m^2$ in untreated cultures (control); cultures treated with 50 μg/ml IgG, 50 μg/ml nrxΔ4-Fc, or 50 μg/ml nrxspl4-Fc were quantified as described in Experimental Procedures (G). The background level of synapsin clustering obtained from a culture without any granule cells is indicated by a broken line (normalized to the average number of cells counted per field for the other conditions). The scale bar in (A) represents 10 μm.

no significant effect (Figure 6D). This suggests that the activity of neuroligin is mediated by its neurexin binding site.

Soluble β-Neurexin Inhibits Vesicle Clustering Induced by a Postsynaptic Target Cell

To assay whether neuroligin is part of the postsynaptic machinery that mediates presynaptic vesicle clustering upon contact of two neuronal cell types, we tested the effect of nrxΔ4-Fc on a coculture of pontine explants with cerebellar granule cells, the in vivo target of the pontine axons. To visualize vesicle clustering exclusively within the pontine axons, but not granule cell axons, we used synapsin as a vesicle marker and isolated granule cells from synapsin-deficient mice (Rosahl et al., 1995). This system has been previously used successfully to compare quantitatively the synaptogenic activity of different target cells (D. E. Emerling, S. Y. Choi, and T. S., submitted). Using this assay, we observed a 45% ± 6% reduction of the number of synapsin clusters induced in pontine axons when 50 μg/ml of nrxΔ4-Fc was added (Figure 7). A control IgG had no significant effect when used at the same concentration. Since the inhibition we observed could have been due to an activity of neurexin other than its neuroligin binding activity, we also tested a splice variant of neurexin fused to IgG (nrxspl4-Fc). This splice variant contains a 30 amino acid insertion in the G domain that abolishes interaction with neuroligin (Ichtchenko et al., 1995). This splice variant did not inhibit vesicle clustering in our assay (Figure 7G). Neither the growth of the pontine axons nor the

morphological maturation of the granule cells were significantly affected by addition of either neurexin-Fc fusion protein (Figures 7A–7F). Therefore, a splice site-specific ligand of β-neurexin, most likely neuroligin, is directly involved in the initial formation or stabilization of presynaptic structure.

Discussion

We have demonstrated that neuroligin-1 can trigger the development of presynaptic structure in the axons of CNS neurons. In particular, (1) contact with neuroligin-expressing, nonneuronal cells leads to the clustering of synaptic vesicles within axons, (2) these contact sites display several functional and morphological hallmarks of neuron–neuron synapses, (3) the activity of neuroligin-1 requires specific sequences in the extracellular, acetylcholinesterase-homologous domain of the protein, (4) neuroligin activity is inhibited by addition of soluble β-neurexin, and (5) addition of soluble β-neurexin inhibits vesicle clustering in axons contacting bona fide target neurons.

Specificity of Neuroligin Function

Previous studies have demonstrated that cationic agents such as synthetic, polylysine-coated beads can induce the development of presynaptic structures in CNS axons (Burry, 1982). This effect is likely to be due to the ability of such an agent to bind and cluster negatively charged molecules through relatively nonspecific, electrostatic interactions.

Several lines of evidence strongly suggest that the activity of neuroligin we observe is the result of specific interactions. The extracellular domain of neuroligin is negatively charged and it is therefore unlikely to act as a polycation. More importantly, we have been able to take advantage of the homology between acetylcholinesterase and neuroligin to address the issue of specificity. Modeling studies suggest that both proteins have very similar electrostatic surface potentials (Botti et al., 1998). Similarity between the two proteins is further supported by studies that reported a functional redundancy for these two proteins in supporting neurite outgrowth in PC12 cells in vitro (Grifman et al., 1998). In our assay, however, replacement of the neuroligin esterase domain with the homologous domain from mouse acetylcholinesterase completely abolishes the activity of neuroligin to induce presynaptic development (Figure 6). Furthermore, a soluble neurexin-IgG fusion protein specifically blocks vesicle clustering induced by neuroligin. All of this evidence strongly suggests that the ability of neuroligin to induce presynaptic development is the result of specific molecular interactions and not nonspecific, electrostatic properties.

Mechanisms of Neuroligin-Induced Synaptic Vesicle Clustering

We have demonstrated that neuroligin's activity only requires extracellular sequences. Therefore, neuroligin is likely to act directly on contacting axons through its extracellular domain, rather than indirectly, through cytoplasmic interactions, or by altering gene expression in HEK293 cells. We cannot completely exclude the possibility that the neuroligin esterase domain might cooperate with an endogenous, cell-surface molecule in order to induce presynaptic development; however, such a molecule would need to be common to all the different cell types that we have tested.

Since the ability to induce presynaptic development strictly correlated with the ability to bind neurexin-1β and could be blocked specifically by addition of soluble neurexin-1β, a β-neurexin might be the active neuroligin binding partner in the axonal plasma membrane. A model explaining the vesicle clustering, but not necessarily other aspects of presynaptic development we observe, is that previously proposed by Südhof and colleagues (Butz et al., 1998). Neuroligin in a target neuron could bind β-neurexin in the presynaptic, axonal membrane. A series of protein–protein interactions that have so far been delineated biochemically could then lead to the recruitment of synaptic vesicles: via its cytoplasmic tail, β-neurexin could either interact directly with the synaptic vesicle protein synaptotagmin, or with CASK/Lin-2 (Hata et al., 1996, 1993); this is consistent with our result that CASK/Lin-2 localizes to the induced presynaptic specializations (Figure 3). CASK/Lin-2 is found in a cytosolic complex with Lin-7 and Lin-10 and this complex has been suggested to provide a link to vesicle exocytosis (Borg et al., 1998; Butz et al., 1998).

However, there are several caveats. As immunological reagents directed specifically against β-neurexins have so far proven elusive, we were unable to test whether a β-neurexin is localized to the presynaptic specializations and is the sole, active binding partner on the axon. Furthermore, we do not observe a concentration of neuroligin opposite synaptic vesicle clusters in our assay (although transient expression might lead to levels of neuroligin on the cell surface similar to that which might occur upon interaction with a ligand). Among CASK/Lin-2, Lin-7, and Lin-10, only CASK/Lin-2 has been localized by electron microscopy to both the pre- and postsynaptic compartments (Hsueh et al., 1998). In contrast, Lin-7 and Lin-10 have been suggested to be localized postsynaptically (Borg et al., 1998; Jo et al., 1999), and functional studies in *Caenorhabditis elegans* have revealed only postsynaptic defects in *lin-10* mutants, whereas the presynaptic distribution of the vesicle marker VAMP2 is unaffected in such mutants (Rongo et al., 1998). Of course, it is possible that CASK/Lin-2 could be part of two different complexes, one presynaptic and one postsynaptic.

Induction of Presynaptic Differentiation by Neuroligin

Neuroligin-1-deficient mice have been reported to be "viable and fertile" (Song et al., 1999). However, we have demonstrated that neuroligin-1 and -2 have comparable activities in our in vitro assay (Figure 2), and our in situ analysis demonstrated that neuroligin-1 and -2 show similar expression throughout the neonatal brain. They are coexpressed in cerebellar Purkinje and granule cells at the developmental stage when these neurons are forming synapses. This suggests that the lack of an obvious phenotype in neuroligin-1-deficient mice could be explained by a functional redundancy of neuroligin-1

and -2. The widespread expression and comparable activities of different neuroligin isoforms and splice variants also suggest that neuroligins are unlikely to specify neuronal cell types for synapse formation, but are more likely to be components of a general synaptogenic machinery.

To test whether neuroligins and neurexins are required during the initial stages of presynaptic differentiation in CNS neurons, we added a soluble neurexin-Fc fusion protein to a coculture of granule cells and pontine explants. This protein reduced synaptic vesicle clustering in pontine axons by 45%, whereas a neurexin splice variant (having a small 30 amino acid insert) that does not bind neuroligin did not show any inhibition (Figure 7G). Although we cannot completely exclude that nrxΔ4-Fc might inhibit vesicle clustering by binding to another, yet unknown splice-site specific ligand of neurexin, it is most likely that it acts by binding to neuroligin. There are several possibilities for why we obtained only a partial inhibition of clustering. First, disrupting neuroligin function with a soluble molecule might not be straightforward, as interactions occur between two closely apposed neuronal membranes brought together by other molecules. Second, we used only part of the neurexin-1 ectodomain, which might have lower affinity for the neuroligins present in granule cells compared to the β-neurexin on the pontine axons. Third, several additional secreted or membrane-bound factors in the granule cells might also drive the initial formation and stabilization of synaptic contacts, which would not be affected by addition of soluble β-neurexin (Hall et al., 2000).

As a postsynaptic, transmembrane protein that can directly induce presynaptic specializations via its extracellular domain, neuroligin is especially well suited to fulfill the role of a retrograde signaling molecule. Neuroligin expression persists in the adult (Song et al., 1999) and could then contribute to remodeling processes as they occur during activity-dependent changes at the synapse (Martin and Kandel, 1996). In addition to a role in the initial formation of synaptic contacts, neuroligin might therefore be employed to regulate the size or strength of synaptic connections, either by inducing new presynaptic specializations or by stabilizing preexisting structures.

Experimental Procedures

DNA Constructs

Murine neuroligin-1 and -2, and neurexin-1β cDNAs were cloned from purified cerebellar granule cells by RT-PCR. Primers were designed based upon the published rat sequences. For expression of neuroligin-1 and -2 in HEK293 cells, an influenza hemagglutinin (HA) tag was inserted between the sequences encoding the signal peptide and the mature N terminus of each of the proteins, and cDNAs were transferred to an expression vector with a CMV promoter (pNICE; gift of J. White, unpublished). In the esterase-homology domain deletion mutant NLGΔAChE, the amino acids 48–630 of murine neuroligin-1*ab* were excised (the HA epitope is followed by the amino acids GVPHLHN). To delete all predicted O-glycosylation sites in the mutant NLGΔO-glyc, amino acids 636–685 were removed. In the mutant NLGAChEswap, amino acids 48–630 of neuroligin-1*ab* were replaced by amino acids 31–565 of mouse acetylcholinesterase (junction with HA epitope: PDYA/AEGR and on the C-terminal side: FWNR/LVPH; the acetylcholinesterase cDNA was provided by P. Taylor). In the mutant swap184, only the C-terminal 184 amino acids of the esterase-homology domain were replaced by acetylcholinesterase sequence (junctions: YGYP/QASD and FWNR/LVPH). In the C-terminal deletion constructs NLGΔC1 and NLGΔC2, a stop codon was inserted after amino acid 803 or amino acid 771, respectively. In NLG/CD8, the cytoplasmic tail of neuroligin-1*ab* was replaced by the cytoplasmic tail of CD8.

The expression constructs for HA-tagged ephrinB1, for rat agrin0 and rat agrin12.4.8 (Ferns et al., 1993), and for TAG-1 were provided by J. P. Labrador, M. Ferns, and M. Tessier-Lavigne, respectively. Mouse N-cadherin (Miyatani et al., 1989) (cDNA provided by M. Takeichi) was fused to a sequence encoding the HA epitope.

Recombinant Proteins

Soluble neurexin-1β (amino acids 47–258 of the mouse protein sequence lacking splice insertion 4) was expressed in *E. coli* (BL21) fused at the N terminus to glutathione-S-transferase (GST) and on the C terminus to a hexa-histidine tag. After affinity purification on glutathione resin, the GST-moiety was cleaved off by TEV-protease, and neurexin was purified to electrophoretic homogeneity on nickel agarose. An expression construct encoding either neurexin-1β-IgG fusion protein (lacking or containing splice insertion 4, amino acids 1–272, or amino acids 1–302, respectively, fused to human IgG sequence) was transiently transfected in HEK293 cells and the secreted fusion protein was purified on a ProteinA HiTrap column (Pharmacia).

A recombinant baculovirus encoding the neuroligin-1*ab* ectodomain with an N-terminal HA tag and C-terminal hexa-histidine tag was constructed using the pFastBac system (GIBCO-BRL) and neuroligin was purified on nickel-agarose (Quiagen). This recombinant protein did not significantly inhibit vesicle clustering in pontine axons, did not block presynaptic development in a coculture of pontine explants with neuroligin-expressing HEK293 cells, and did not bind neurexin in in vitro assays (data not shown). This protein might lack activity, either as a result of expression in a heterologous system and/or the lack of a membrane anchor.

Antibodies

Antibodies against neuroligin-1 were raised in rabbits using a peptide with an N-terminal cysteine added for coupling (CKQDDPKQQ PSPFSVDQRDYST). The polyclonal rabbit antibody against the luminal domain of synaptotagmin was raised against a synthetic peptide (MVSASRPEALAC). Mouse monoclonal antibodies Cl 41.1 anti-synaptotagmin (Brose et al., 1992) and C 7.2 anti-synaptophysin (Jahn et al., 1985) were provided by R. Jahn; mouse monoclonal IgM 4D7 anti-TAG-1 was provided by M. Tessier-Lavigne. The following commercially available antibodies were used: rabbit and mouse anti-synapsin I, mouse anti-CASK (Chemicon), mouse and rat anti-HA-tag, mouse anti-His_6, (Boehringer Mannheim), mouse anti-agrin (Stressgen), and mouse anti-tubulin (Amersham). Secondary antibodies were from Jackson ImmunoResearch, except the 15 nm gold-conjugated goat anti-rat (BBI International).

Cell Culture Assays

HEK293 and COS-7 cells were cultured according to standard procedures. For cocultures with neurons, the cells were seeded at a density of 74,000 cells/cm^2 in Lab-Tek Permanox culture chambers coated with 10 μg/ml polyornithine (Sigma) and 30 μg/ml mouse EHS laminin (Boehringer Mannheim) for 2 hr each. After 24 hr cells were transfected using Lipofectamine Plus (Life Technologies). The medium was exchanged to neuronal culture medium (Neurobasal medium, B27 supplement, Glutamax, penicillin, streptomycin, and 5 ng/ml BDNF), and pontine explants dissected from mice (postnatal days 0.5–1 [P0.5–1]) or cerebellar granule cells (62,000 granule cells per cm^2) were added. After 24–48 hr of coculture the cells were fixed and processed for immunohistochemistry. Cerebellar granule cells and astroglia were purified from P5-P7 mice essentially as described (Hatten, 1985; Baird et al., 1992). Granule cell pontine explant coculture assays were performed as described (D. E. Emerling, S. Y. Choi, and T. S., submitted). In brief, granule cells isolated from P5 or P6 synapsin KO animals (Rosahl et al., 1995) were plated at 62,000 granule cells per cm^2 and cultured for 48 hr. Recombinant proteins were added to the culture medium as indicated and pontine explants dissected from P0.5 wild-type mice were placed in the culture dish.

After 48 hr of coculture, the cells were processed for immunohistochemistry and stained with antibodies anti-synapsin and anti-MAP2. None of the recombinant proteins significantly affected growth of the axons extending from the explants or the morphological maturation of granule cells.

To assay for vesicle turnover, cultures were incubated for 5 min with a prewarmed, isotonic depolarization solution containing 10 μg/ml of affinity-purified rabbit anti-synaptotagmin luminal domain antibodies. A control rabbit IgG was used at the same concentration. After incubation, cultures were briefly washed 3 times with prewarmed medium, fixed, and processed for immunohistochemistry.

Image Acquisition and Quantitation

Images were taken with a Leica TCS NT confocal microscope (Leica, Heidelberg, Germany). Laserpower and photomultipliers were set such that no detectable bleedthrough occurred between the different channels. Eight sections were taken from top to bottom of the specimen and brightest point projections were made. Images were processed using Photoshop software (Adobe Systems, CA).

In the experiments to localize CASK/Lin-2 to axonal structures, thin confocal slices were taken using a 100× lens and the zoom function of the Leica TCS confocal microscope. This allowed us to separate CASK/Lin-2 immunoreactivity in axons (identified by overlap with synapsin staining) from CASK/Lin-2 inside the HEK293 cells. No significant concentration of CASK/Lin-2 was observed within the HEK293 cells, but strong immunoreactivity within the axons was observed (data not shown).

For quantitation of synapsin accumulation in pontine axons, random images were collected with identical confocal settings at a 100–400 μm distance from the explant using a 40× lens. Explants with assymetric or limited growth were excluded from the analysis. Using OpenLab software (Improvision), density slices of synapsin staining signal above a threshold were taken to create binary images. For the pontine explant-HEK293 cocultures, this threshold was defined as a staining intensity 5-fold higher than synapsin staining in isolated axons in a control sample. All pixels above the threshold were scored and normalized to the number of transfected HEK293 cells in the analyzed field (2–10 cells, similar for the different transfected cDNAs analyzed). For each quantitation, at least 12 images per transfected cDNA were scored, and the mean and standard error were calculated. Several independent quantitations yielded similar results.

To quantitate the number of presynaptic specializations in a coculture of pontine explants with granule cells, the images were collected as described above. Presynaptic vesicle clusters were detected by synapsin immunostaining and are characterized by their size and staining intensity. Density slices of synapsin staining above an intensity threshold were taken and the number of objects equal to or larger than 1 μm^2 was determined for the entire field and then normalized to the number of granule cells (detected by immunostaining for MAP2) in the field. Under these conditions, 93% ± 1.5% of all objects scored as vesicle clusters were directly associated with MAP2 positive structures. Results were averaged for at least ten images for each condition (with 10–20 granule cells per field) and the mean and standard error for the number of clusters per granule cell were calculated. Several independent experiments and quantitations yielded similar results.

Vesicle turnover in pontine axons was quantified essentially as described (Kraszewski et al., 1995). In brief, confocal scans were performed and for recognizable spots on targets, the average pixel intensity in a rectangle of 5 × 5 μm was calculated for the Cy3 and Cy5 channels, representing the staining for internalized synaptotagmin luminal domain antibodies and total synaptotagmin detected by the cytoplasmic domain antibodies, respectively.

Neurexin Binding Experiments

HEK293 cells transiently transfected with neuroligin expression constructs were incubated with 1 μg/ml hexa-histidine-tagged neurexin-1β for 30 min at 4°C in α-MEM / 0.2% BSA / 25 mM HEPES. When desired, free calcium was complexed by addition of 10 mM EGTA to the incubation buffer. The cells were washed twice, fixed and processed for immunohistochemistry using antibodies against the HA epitope and the hexa-histidine tag to detect neuroligin and neurexin, respectively.

Electron Microscopy

HEK293 cells were cultured on Aclar film (Ted Pella, Inc.) and transfected as above. After 48 hr of coculture with pontine explants, live cultures were labeled at 37°C with rat anti-HA (0.5 μg/ml) and secondary goat anti-rat 15 nm gold-conjugated antibodies (0.18 μg/ml). Cells were then processed for electron microscopy. The specimens were fixed for 1 hr with 2% glutaraldehyde in cacodylate buffer (0.1 M Na-cacodylate [pH 7.4]) and post-fixed with 1% osmium tetroxide in cacodylate buffer. The osmicated samples were stained en bloc with 1% uranyl acetate in distilled water and dehydrated in ethanol followed by propylene oxide. The specimens were then infiltrated and embedded in Epon resin. Serial sections were cut with a diamond knife using a Reichert-Jung Ultracut E ultramicrotome and stained with uranyl acetate and Sato's lead (Sato, 1968). Sections were examined and photographed with a JEOL 1200EX/II electron microscope operated at 80 kV.

In Situ Hybridizations

Two antisense probes were used for detection of neuroligin-1 mRNA. The 3′-untranslated region of mouse neuroligin-1 was amplified by RT-PCR using nested sense oligonucleotides from the 3′ end of the coding region and oligo dT as primers. As a second probe, an 828 bp PstI-KpnI fragment encoding part of the neuroligin-1 ectodomain was used. This fragment shares 72% sequence identity with neuroligin-3, and 71% identity with neuroligin-2, but no significant homology with esterase sequences. Both neuroligin-1 probes gave identical results. As a neuroligin-2 specific probe, a 413 bp BspHI-XhoI fragment was used, encoding part of the esterase domain and the O-glycosylated stralk region, sharing 72% identity with neuroligin-3 and 71% identity with neuroligin-1. RNA in situ hybridization was carried out as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Barth et al., 1997) with minor modifications. Antisense RNA probes were synthesized from linearized templates and hybridized to slides at 10^7 cpm/ml for ^{35}S-labeled probes or 0.7 μg/ml for DIG-labeled probes at 72°C overnight, and washed in 0.2×SSC at 72°C. Slides hybridized with ^{35}S-labeled probes were exposed to Kodak NTB-2 emulsion, and slides hybridized with DIG-labeled probes were visualized with an alkaline-phosphatase-conjugated anti-DIG F(ab) and NBT/BCIP. These hybridization conditions allow transcripts of sequence identity of 75%–80% to be distinguished unambiguously (Barth et al., 1997).

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