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Nectin: an adhesion molecule involved in formation of synapses

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The nectin–afadin system is a novel cell–cell adhesion system that organizes adherens junctions cooperatively with the cadherin–catenin system in epithelial cells. Nectin is an immunoglobulin-like adhesion molecule, and afadin is an actin filament–binding protein that connects nectin to the actin cytoskeleton. Nectin has four isoforms (-1, -2, -3, and -4). Each nectin forms a homo-cis-dimer followed by formation of a homo-trans-dimer, but nectin-3 furthermore forms a hetero-trans-dimer with nectin-1 or -2, and the formation of each hetero-trans-dimer is stronger than that of each homo-trans-dimer. We show here that at the synapses between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area of adult mouse hippocampus, the nectin–afadin system colocalizes with the cadherin–catenin system, and nectin-1 and -3 asymmetrically localize at the pre- and postsynaptic sides of puncta adherentia junctions, respectively. During development, nectin-1 and -3 asymmetrically localize not only at puncta adherentia junctions but also at synaptic junctions. Inhibition of the nectin-based adhesion by an inhibitor of nectin-1 in cultured rat hippocampal neurons results in a decrease in synapse size and a concomitant increase in synapse number. These results indicate an important role of the nectin–afadin system in the formation of synapses.

Introduction

Synapses have been regarded as one of the specialized cell–cell junctions whose specificity and plasticity provide neurons with a structural and functional basis for neural network formation. The molecular architecture of the cell–cell junctions of synapses is, however, poorly understood. At least two types of cell–cell junctions with different functions have been recognized at synapses: synaptic and puncta adherentia junctions (Peters et al., 1976). Synaptic junctions are sites of neurotransmission, consisting of active zones where Ca^{2+} channels localize and synaptic vesicles are docked and fused, and postsynaptic densities where neurotransmitter receptors localize. Puncta adherentia junctions have symmetrical paramembranous dense materials and no association with synaptic vesicles or postsynaptic densities, and appear to be ultrastructurally similar to adherens junctions of epithelial cells. They are regarded as mechanical adhesion sites between axon terminals and their target cells, but their molecular mechanisms remain largely unknown. At the synapses between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area of hippocampus, both synaptic and puncta adherentia junctions are highly developed. These junctions are actively remodeled in an activity-dependent manner during development of the brain, even in adulthood. The formation and remodeling of these junctions in this area has been suggested to play a key role in synaptic plasticity, a principal mechanism of memory and learning.

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*Abbreviations used in this paper: Ab, antibody; CSM, crude synaptic membrane fraction; CSV, crude synaptic vesicle fraction; E-cadherin, epithelial cadherin; gD, glycoprotein D; Ho, the homogenate fraction; N-cadherin, neural cadherin; P1, nuclear pellet fraction; P2, crude synaptosome fraction; P3, microsome fraction; P4, -7, and -14, postnatal day 4, 7, and 14; P2A, myelin fraction; P2B, ER and Golgi complex fraction; P2C, synaptosome fraction; P2D, mitochondria fraction; PSD, postsynaptic density fraction; S, soluble cytosol fraction; SM1–SM3, synaptic membrane fraction; SM4, intrasynaptosomal mitochondria fraction; SS, synaptic soluble fraction.

Similarity in molecular mechanisms between junctions of synapses and epithelial cells has recently been substantiated by the findings that classical cadherins, neural (N)*- and epithelial (E)-cadherins, localize at both synaptic and puncta adherentia junctions in at least some brain regions (Yamagata et al., 1995; Fannon and Colman, 1996), and that αN- and β-catenins, cadherin-associated proteins, also localize at puncta adherentia junctions (Uchida et al., 1996). Furthermore, a novel type of cadherin, cadherin-related neuronal receptor, has been found to localize at synaptic junctions (Kohmura et al., 1998). These findings suggest that both synaptic and puncta adherentia junctions of synapses are cadherin-based cell–cell junctions just as are typical adherens junctions in epithelial cells.

We have recently found a novel cell–cell adhesion system at cadherin-based adherens junctions, consisting of at least two components, nectin and afadin (Mandai et al., 1997; Takahashi et al., 1999). Nectin is a Ca^{2+}-independent cell–cell adhesion molecule that belongs to the immunoglobulin superfamily (Aoki et al., 1997; Lopez et al., 1998; Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Reymond et al., 2001; Sakisaka et al., 2001). Nectin comprises a family consisting of at least four members, nectin-1, -2, -3, and -4. Nectin-1 is identical to the poliovirus receptor–related protein and has recently been shown to serve as the α-herpes virus entry and cell–cell spread mediator (Geraghty et al., 1998; Warner et al., 1998; Cocchi et al., 2000; Sakisaka et al., 2001). Each member of the nectin family forms a homo-cis-dimer, followed by formation of a homo-trans-dimer, causing cell–cell adhesion (Lopez et al., 1998; Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Reymond et al., 2001; Sakisaka et al., 2001). Nectin-3 furthermore forms a hetero-trans-dimer with either nectin-1 or -2, and the formation of each hetero-trans-dimer is stronger than that of each homo-trans-dimer (Satoh-Horikawa et al., 2000). Nectin-4 also forms a hetero-trans-dimer with nectin-1, and this formation is also stronger than that of the homo-trans-dimer (Reymond et al., 2001). Each member of the nectin family except nectin-4 has two or three splicing variants (Morrison and Racaniello, 1992; Eberlé et al., 1995; Lopez et al., 1995; Cocchi et al., 1998; Satoh-Horikawa et al., 2000). Most members have a COOH-terminal conserved motif of four amino acid residues (E/A-X-Y-V) that interacts with the PDZ domain of afadin (Takahashi et al., 1999; Satoh-Horikawa et al., 2000). Nectin-4 does not have the conserved motif, but interacts with the PDZ domain of afadin through its COOH terminus (Reymond et al., 2001). Afadin has at least two splicing variants, l- and s-afadin. l-Afadin, a larger splicing variant that connects nectin to the actin cytoskeleton, is an actin filament–binding protein with one PDZ domain and three proline-rich domains. (Mandai et al., 1997; Takahashi et al., 1999). s-Afadin, a smaller splicing variant, has one PDZ domain, but lacks the actin filament–binding domain and the third proline-rich domain (Mandai et al., 1997). Human s-afadin is identical to the AF6 protein, the gene of which is originally found to be fused to the ALL-1 gene in acute leukemia cases (Prasad et al., 1993).

The nectin-based cell–cell adhesion has a potency to recruit the E-cadherin–catenin complex through l-afadin, and is cooperatively involved with the cadherin–catenin system during the formation of adherens junctions (Takahashi et al., 1999; Miyahara et al., 2000; Tachibana et al., 2000). In epithelial cells of afadin$^{-/-}$ mice and afadin$^{-/-}$ embryoid bodies, the proper organization of adherens junctions is severely impaired (Ikeda et al., 1999). In spermatozoa of nectin-2$^{-/-}$ mice, the nuclear and cytoskeletal morphology and mitochondrial localization are impaired (Bouchard et al., 2000). Thus, accumulating evidence demonstrates the important role of the nectin–afadin system in the organization of adherens junctions.

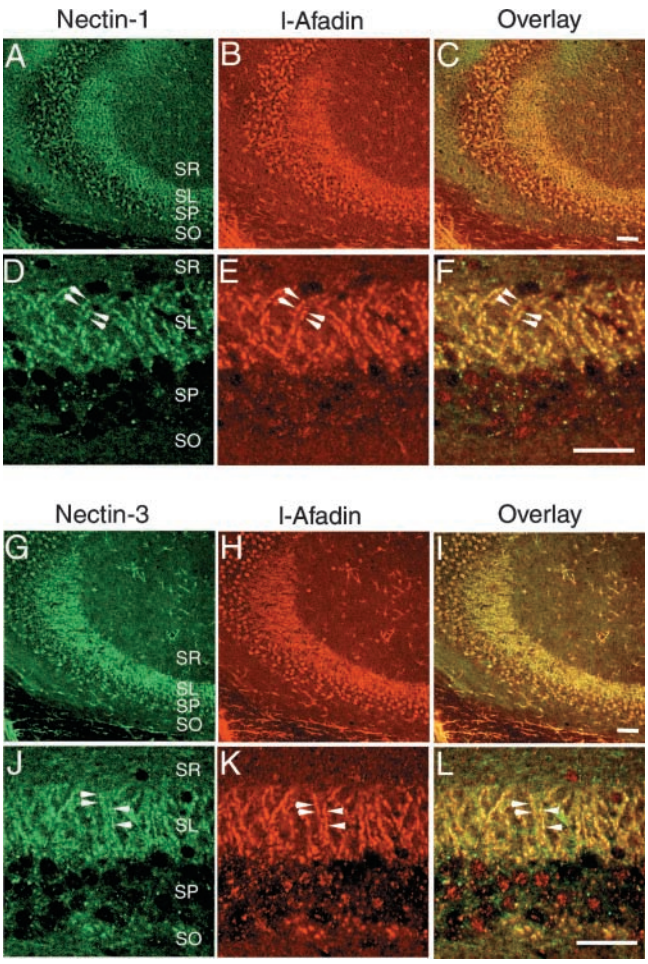


Figure 1. **Colocalization of nectin-1 and -3 with l-afadin at stratum lucidum of adult mouse hippocampus.** Adult mouse hippocampal sections were doubly stained with the rat monoclonal anti–nectin-1 or rat monoclonal anti–nectin-3 and rabbit polyclonal anti–l-afadin Abs, followed by immunofluorescence microscopy. (A–F) Double staining of nectin-1 and l-afadin; (G–L) double staining of nectin-3 and l-afadin; (A–C and G–I) hippocampus at low magnification; (D–F and J–L) CA3 area at high magnification; (A and D) nectin-1; (G and J) nectin-3; (B, E, H, and K) l-afadin; and (C, F, I, and L) overlay. The staining pattern obtained by the rat monoclonal anti–nectin-1 or -3 Ab, which recognized each extracellular domain, was basically the same as that by the rabbit polyclonal Ab, which recognized each cytoplasmic domain, respectively. SL, stratum lucidum; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Arrowheads, small dots aligning along the putative dendritic trunks of pyramidal cells at stratum lucidum. The results shown are representative of three independent experiments. Bars, 30 μm.

s-Afadin/AF6 associates with the Eph B receptor tyrosine kinases, and has been shown to localize at postsynaptic densities of synaptic junctions in the CA1 area of adult rat hippocampus (Hock et al., 1998; Buchert et al., 1999). We have found that l-afadin colocalizes with αN-catenin symmetrically at puncta adherentia junctions of the synapses between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area of adult mouse hippocampus (Nishioka et al., 2000). More recently, nectin-1 has been determined, by positional cloning, to be responsible for cleft lip/palate–ectodermal dysplasia, which is characterized by cleft lip/palate, syndactyly, mental retardation, and ectodermal dysplasia (skin, hairs, nails, teeth, and sweat glands) (Suzuki et al., 2000). Accumulating evidence suggests that the nectin–afadin system, as well as the cadherin–catenin system, plays an important role in the formation and remodeling of synaptic and puncta adherentia junctions. We have examined here whether the nectin–afadin system is involved in the formation of synapses.

Results

Asymmetric localization of nectin-1 and -3 at puncta adherentia junctions

We first studied the localization of nectin-1, -2, and -3 in the CA3 area of adult mouse hippocampus. The most intense immunoreactivity for nectin-1 was demonstrated as dots or flattened disks that aligned along the dendritic trunks of pyramidal cells at stratum lucidum of the CA3 area (Fig. 1, A and D). In addition to the signals at stratum lucidum, moderate immunoreactivity for nectin-1 was also found as smaller dots at strata radiatum and oriens. The immunoreactivity for l-afadin was also detected at stratum lucidum as flattened disks where nectin-1 colocalized with l-afadin almost completely (Fig. 1, B, C, E, and F). In addition to the signals at stratum lucidum, the nuclei of pyramidal cells were positive for l-afadin, although the physiological significance is unknown. Some round or linear signals larger than 10 μm and immunoreactive for both nectin-1 and l-afadin at strata radiatum and oriens were derived from glial cells and blood vessels, as confirmed by immunoelectron microscopy (unpublished data).

The immunoreactivity for nectin-3 was demonstrated as flattened disks at stratum lucidum and as smaller dotty and larger linear images at strata radiatum and oriens (Fig. 1, G and J). This staining pattern of nectin-3 was very similar to that of nectin-1. Nectin-3 also colocalized with l-afadin at stratum lucidum (Fig. 1, H, I, K, and L). Taken together, nectin-1 and -3 colocalized with l-afadin at stratum lucidum as flattened disks that aligned along the dendritic trunks of pyramidal cells. These structures corresponded to the synapses between the mossy fiber terminals and dendrites of pyramidal cells. Nectin-1 and -3 also colocalized with l-afadin at strata radiatum and oriens as the larger linear images that corresponded to some glial cells and blood vessels. However, there were small dotty signals at stratum radiatum that were immunoreactive for nectin-1 or -3 but not for l-afadin. At these l-afadin–deficient areas, nectin-1 and -3 may be associated with s-afadin, a smaller splicing variant of l-afadin, or unidentified peripheral membrane proteins. The immunoreactivity for nectin-2 was undetectable in the CA3 area (unpublished data). It has been shown that nectin-4 is expressed in mouse brain (Reymond et al., 2001), but the localization of nectin-4 in the brain was not examined here because its antibody (Ab) was not available.

Immunoelectron microscopic analysis revealed that ~90% of the immunogold particles detected with the anti–nectin-1 and anti–nectin-3 Abs that were raised against the cytoplasmic domains of nectin-1α and -3α, respectively, were concentrated in an asymmetrical manner on the plasma membranes of the mossy fiber terminals (presynaptic side) and dendrites (postsynaptic side) of pyramidal cells, respectively, at puncta adherentia junctions (Fig. 2, A and B). In contrast, the immunogold particles detected by the anti–l-afadin Ab were concentrated on both sides of the junctions (Fig. 2 C). It is therefore likely that at puncta adherentia junctions of these synapses,

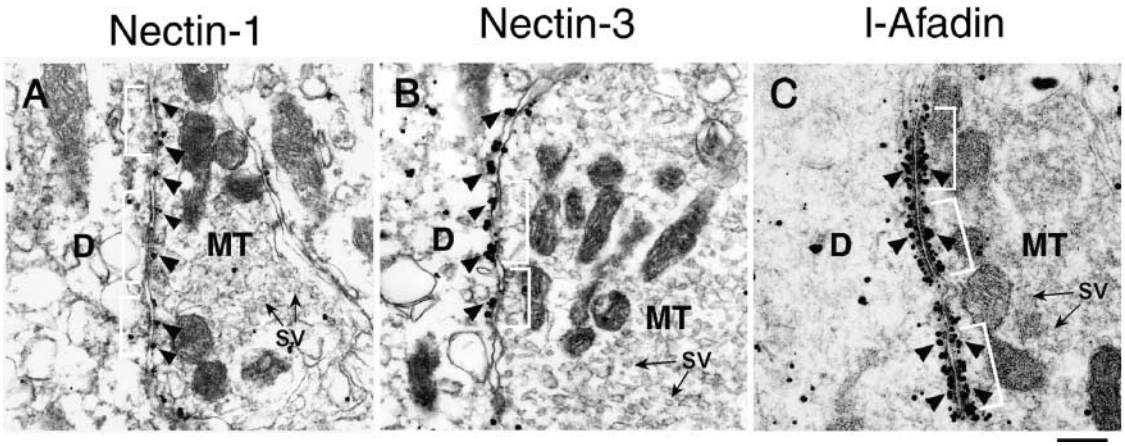


Figure 2. **Asymmetric localization of nectin-1 and -3 at the pre- and postsynaptic sides of puncta adherentia junctions.** Adult mouse hippocampal sections were singly stained with the rabbit polyclonal anti–nectin-1, rabbit polyclonal anti–nectin-3, or rabbit polyclonal anti-l-afadin Ab, followed by immunoelectron microscopy with the silver-enhanced immunogold method. (A) Nectin-1, (B) nectin-3, and (C) l-afadin. 87% and 92% of immunogold signals for nectin-1 and -3 are seen at the pre- and postsynaptic sides of puncta adherentia junctions between the mossy fiber terminals and dendrites of pyramidal cells, respectively ($n = 12$), whereas those of l-afadin are at both sides. MT, mossy fiber terminals; D, dendritic trunks of pyramidal cells. Arrowheads, immunogold particles; SV, synaptic vesicles; Brackets, puncta adherentia junctions. Bar, 500 nm.

nectin-1 and -3 form a hetero-trans-dimer that is associated with l-afadin on both sides of the junctions. No signal for nectin-1, -3, or l-afadin was detected at synaptic junctions located on the spines of the dendrites (unpublished data).

Enrichment of nectin-1 and -3 in the synaptic membrane fraction

The localization of nectin-1, -3, and l-afadin at synapses was examined in comparison with αN-catenin and N-cadherin by subcellular fraction analysis of rat brain. The homogenate fraction (Ho) was first fractionated into the nuclear pellet fraction (P1), the crude synaptosome fraction (P2), the microsome fraction (P3), and the soluble cytosol fraction (S). P2 was further fractionated into the myelin fraction (P2A), the ER and Golgi complex fraction (P2B), the synaptosome fraction (P2C), and the mitochondria fraction (P2D). P2C was then fractionated into the crude synaptic membrane fraction (CSM), the crude synaptic vesicle fraction (CSV), and the synaptic soluble fraction (SS). CSM was further fractionated into the synaptic membrane fraction (SM1–SM3) and the intrasynaptosomal mitochondria fraction (SM4), and the postsynaptic density fraction (PSD) was prepared from SM3 with Triton X-100. Nectin-1 and -3 were detected in the synapse-enriched fractions (P2 and P2C), but were more abundant in other fractions, such as P3 and P2B, indicating that these proteins were mainly distributed in areas other than synapses (Fig. 3). These results are apparently inconsistent with those obtained by immunofluorescence microscopy (Fig. 1). However, the distribution of nectin-1 and -3 in fractions other than P2 and P2C may be due to their expression in cells other than neurons, such as glial cells and blood vessels. It is possible that nectin-1 and -3 are mainly distributed in these types of cells throughout the whole brain. In addition to the synapse-enriched fractions (P2 and P2C), l-afadin, αN-catenin, and N-cadherin were also distributed in other fractions, such as P1, P3, P2A, and P2B. Of all the fractions, nectin-1 and -3 were most enriched in SM1–SM3, but hardly recovered in PSD. In contrast, l-afadin, αN-catenin, and N-cadherin were concentrated in SM1–SM3 and most enriched in PSD. The reason why nectin-1 and -3 were hardly recovered in PSD is not known, but these proteins may not be tightly associated with postsynaptic densities or active zones. It may be noted that nectin-1 and N-cadherin were detected in P2A, whereas l-afadin and αN-catenin were not. Nectin-1 and αN-cadherin in P2A may not be associated with l-afadin and αN-catenin, respectively.

Changes in distribution of nectin-1, -3, and l-afadin during developmental domain segregation of synaptic and puncta adherentia junctions

It has been shown that the synapses between the mossy fiber terminals and dendrites of pyramidal cells are postnatally formed and remodeled to maturated synaptic and puncta adherentia junctions (Amaral and Dent, 1981). Therefore, we analyzed the localization of nectin-1, -3, and l-afadin in comparison with that of αN-catenin and N-cadherin during development of the CA3 area of mouse hippocampus. On postnatal day 4 (P4), when very immature synapses are formed at stratum lucidum, nectin-1, -3, l-afadin, αN-catenin, and N-cadherin colocalized as small dots at immature synapses (Fig. 4 A). At later stages such as on P7 and P14, these molecules colocalized at the synapses of the stratum lucidum.

As observed by immunoelectron microscopy, on P4, nectin-1 and -3 localized asymmetrically at the pre- and postsynaptic sides of cell–cell contact sites, respectively (Fig. 4 B). l-Afadin and αN-catenin were detected symmetrically at both sides of the immature contact sites, where discrimination between synaptic and puncta adherentia junctions was not clearly observed yet. On P7, when synaptic junctions are clearly identified by prominent postsynaptic densities, nectin-1 and -3 localized asymmetrically at the pre- and postsynaptic sides not only of puncta adherentia junctions but also of synaptic junctions. Again, l-afadin and αN-catenin localized symmetrically at both sides of synaptic and puncta adherentia junctions. On P14, when synaptic and puncta adherentia junctions locate exclusively on the spines and dendritic shafts, respectively, nectin-1 and -3 localized selectively at puncta adherentia junctions together with l-afadin and αN-catenin. The localization patterns on P14 were basically the same as those found in adult mouse hippocampus (Fig. 2, A–C). These results indicate that the localization patterns of nectin-1, -3, l-afadin, and αN-catenin

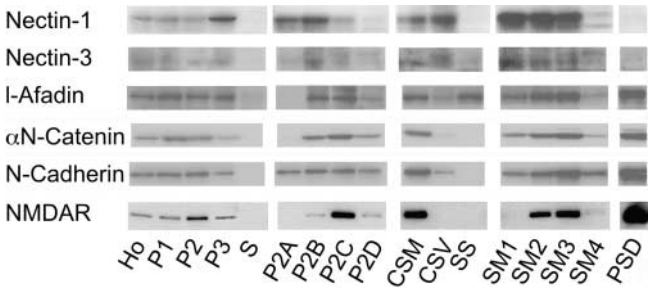
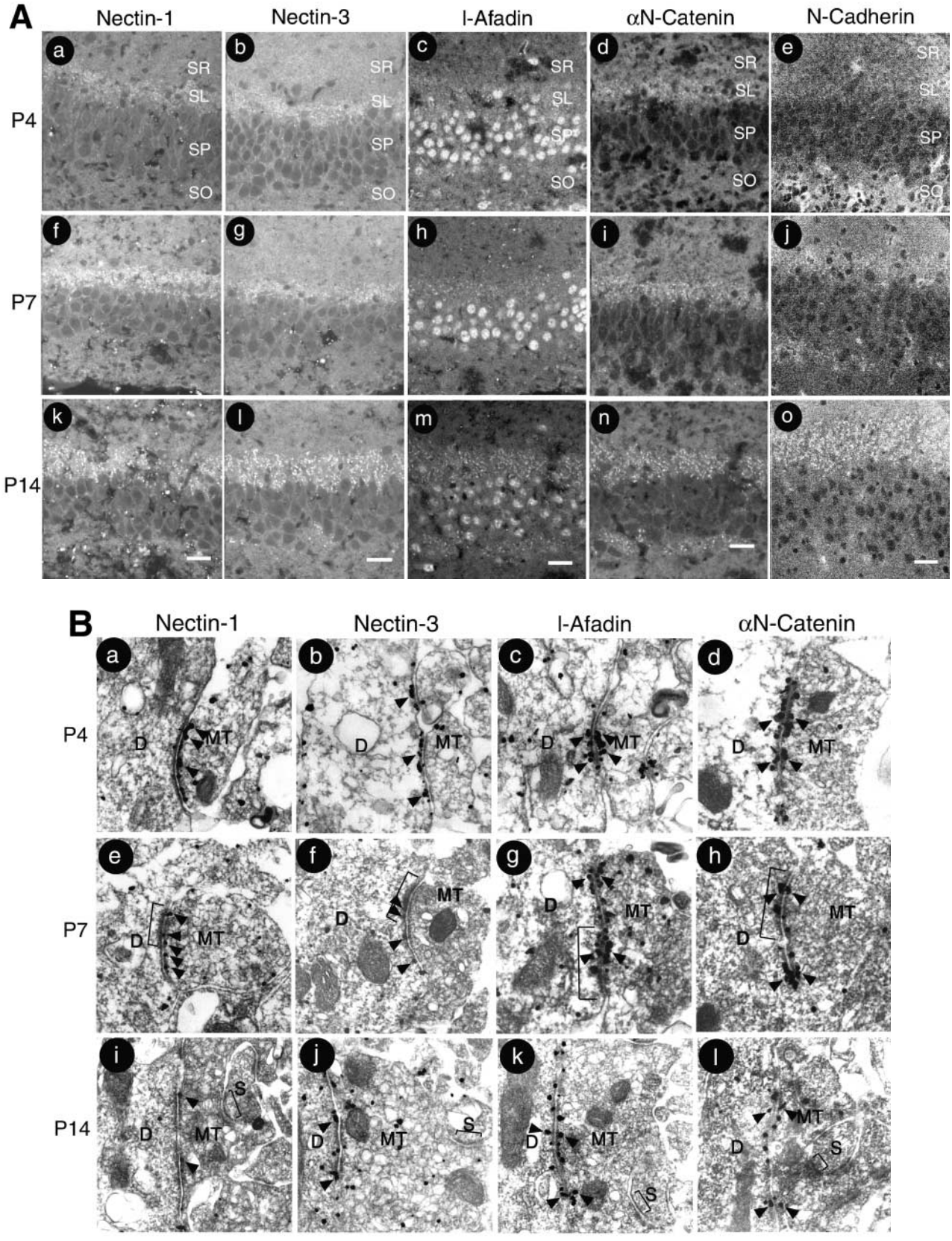


Figure 3. **Enrichment of nectin-1 and -3 in the synaptic membrane fraction of rat brain.** Rat brains were homogenized, followed by subcellular fractionation by differential centrifugation as previously described (Mizoguchi et al., 1990). Ho was first fractionated into P1, P2, P3, and S. P2 was then fractionated into P2A, P2B, P2C, and P2D. P2C was fractionated into CSM, CSV, and SS. CSM was further fractionated into SM1–SM4. PSD was prepared from SM3 with Triton X-100. Each subcellular fraction (10 μg of protein) was subjected to SDS-PAGE (10% gel), followed by Western blotting using the rabbit polyclonal anti–nectin-1, rabbit polyclonal anti–nectin-3, rabbit polyclonal anti–l-afadin, rat monoclonal anti–αN-catenin, mouse monoclonal anti–N-cadherin, and rabbit polyclonal anti–*N*-methyl-D-aspartate receptor Abs. The results shown are representative of three independent experiments.

Figure 4. **Localization of nectin-1, -3, l-afadin, and αN-catenin in stratum lucidum of developing mouse hippocampus.** Developing mouse hippocampal sections on P4, P7, and P14 were singly stained with the rat monoclonal anti–nectin-1, rat monoclonal anti–nectin-3, rabbit polyclonal anti–l-afadin, rat monoclonal anti–αN-catenin, or mouse monoclonal anti–N-cadherin Ab, followed by immunofluorescence microscopy. Alternatively, the sections were singly stained with the rabbit polyclonal anti–nectin-1, rabbit polyclonal anti–nectin-3, rabbit polyclonal anti–l-afadin, or rat monoclonal anti–αN-catenin Ab, followed by immunoelectron microscopy with the silver-enhanced immunogold method. (A) Immunofluorescence microscopy. (a–e) P4; (f–j) P7; (k–o) P14; (a, f, and k) nectin-1; (b, g, and l) nectin-3; (c, h, and m) l-afadin; (d, i, and n) αN-catenin; and (e, j, and o) N-cadherin. The results shown are representative of three independent experiments. (B) Immunoelectron



microscopy. (a–d) P4; (e–h) P7; (i–l) P14; (a, e, and i) nectin-1; (b, f, and j) nectin-3; (c, g, and k) l-afadin; and (d, h, and l) αN-catenin. On P7, 37, 34, 41, and 33% of signals for nectin-1, and -3, l-afadin, and αN-catenin, respectively, are seen on synaptic junctions (brackets). On P14, synaptic junctions with postsynaptic densities (brackets) are seen exclusively on the spines where less than 5% of signals for these molecules are detected. Signals for these molecules are seen almost exclusively at puncta adherentia junctions ($n = 5$; 36–64 synapses at each stage). D, dendritic trunks of pyramidal cells; MT, mossy fiber terminals; SL, stratum lucidum; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Arrowheads, immunogold particles; Brackets, synaptic junctions. Bars: (A) 30 μm; (B) 500 nm.

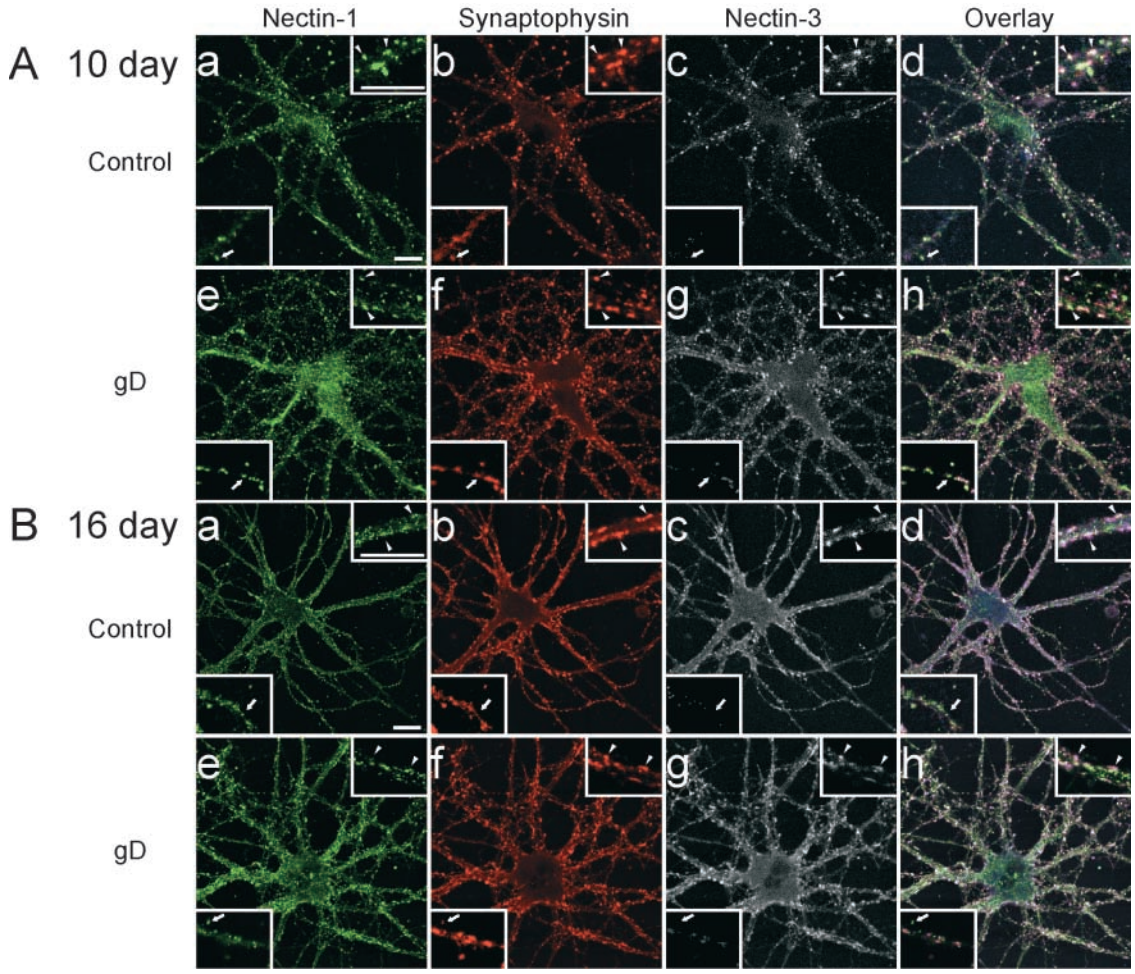


Figure 5. **Localization of nectin-1 and -3 at developing synapses in cultured hippocampal neurons, and impairment of the formation of synapses by inhibition of the nectin-based adhesion.** Rat hippocampal neurons were cultured in the presence of intact gD or boiled gD (control). The neurons were then triply stained with the rabbit polyclonal anti–nectin-1, mouse monoclonal anti-synaptophysin, and rat monoclonal anti–nectin-3 Abs, followed by immunofluorescence microscopy. (A) 10 d in culture; (B) 16 d in culture. (a–d) In the presence of 70 μg/ml of boiled gD; (e–h) in the presence of 8.75 μg/ml of gD; (a and e) nectin-1 (green); (b and f) synaptophysin (red); (c and g) nectin-3 (white); and (d and h) overlay of nectin-1 (green), synaptophysin (red), and nectin-3 (blue). Arrowheads in upper right insets are pointing to synapses (puncta immunoreactive for nectin-1, -3, and synaptophysin), and arrows in lower left insets are pointing to varicosities (puncta immunoreactive for nectin-1 and synaptophysin but not for nectin-3). The results shown are representative of three independent experiments. Bars, 30 μm.

are well correlated with the formation and maturation of synapses at least in the CA3 area. Although, a few signals of nectin-1 and -3 were detected at random in the pre- and postsynaptic sides, it is not known whether these signals have any physiological significance. One possible explanation is that they localize on vesicles where nectin-1 and -3 are transported or recycled.

Localization of nectin-1 and -3 at developing synapses in cultured hippocampal neurons and impairment of formation of synapses by inhibition of the nectin-based adhesion

To obtain the evidence that the nectin–afadin system is indeed involved in the formation of synapses, we added an inhibitor of nectin-1, glycoprotein D (gD), and examined the formation of synapses microscopically. gD is an envelope protein of herpes simplex virus type 1 that binds to nectin-1 and partially inhibits not only the formation of a homo-trans-dimer of nectin-1 but also the formation of a hetero-trans-dimer between nectin-1 and -3 (Sakisaka et al., 2001; unpublished data). We analyzed by immunofluorescence microscopy the localization of nectin-1 and -3, with synaptophysin as a synaptic vesicle marker protein (Navone et al., 1986) and PSD-95 as a postsynaptic marker protein (Cho et al., 1992) in primary cultured rat hippocampal neurons. After 10 d in culture when the number of cell–cell contact sites increased, the nectin-1 signal localized at puncta and ~70% of these puncta were immunoreactive for synaptophysin (Fig. 5 A). Nectin-3, furthermore, colocalized at two thirds of the puncta immunoreactive for both nectin-1 and synaptophysin. The puncta immunoreactive for nectin-1, synaptophysin, and nectin-3 likely correspond to synapses (Fig. 5 A, arrowheads in upper right insets). Consistently, most of the nec-

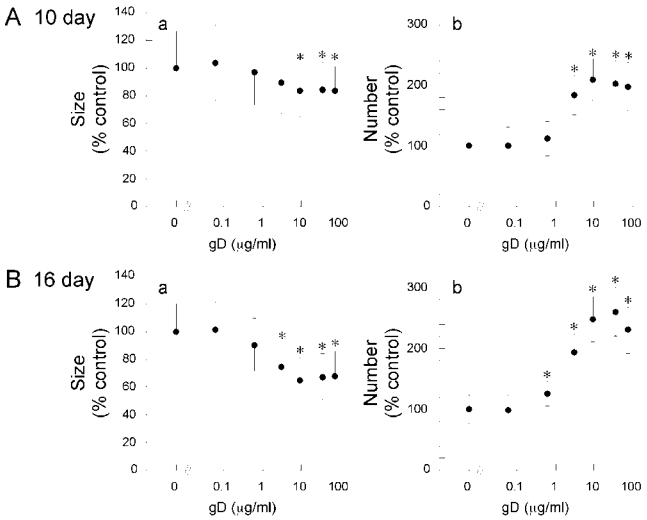


Figure 6. **Quantitative analysis of the effects of gD on the formation of synapses in cultured hippocampal neurons.** Rat hippocampal neurons were cultured in the presence of various concentrations of intact gD or 70 μg/ml of boiled gD (control). The puncta immunoreactive for nectin-1, -3, and synaptophysin were counted as synapses and their sizes were measured. The average size and number of synapses were plotted using the public-domain NIH image program v1.61. (A) 10 d in culture; (B) 16 d in culture. (a) Average size; (b) average number. Values are means ± SEM. $*P < 0.01$. The results shown are representative of three independent experiments.

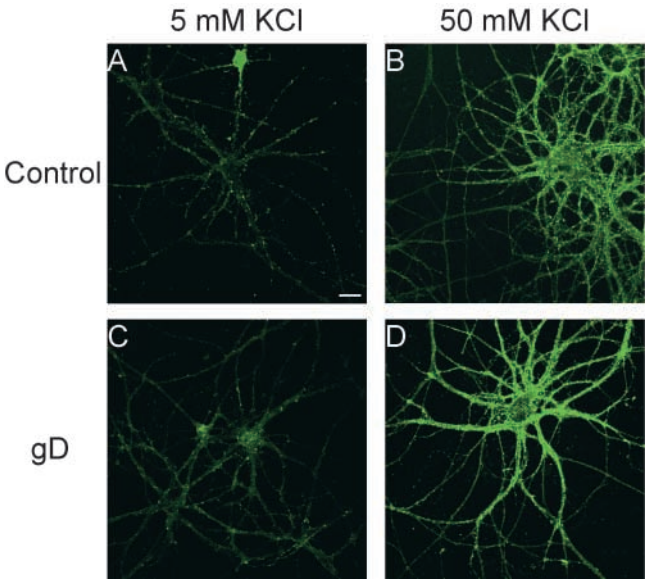


Figure 7. **Vesicle recycling in response to high K^+ stimulation.** Rat hippocampal neurons were cultured in the presence of 35 μg/ml of intact gD or boiled gD (control). After 16 d in culture, the neurons were incubated with 5 or 50 mM KCl and 20 μM FM1-43 for 90 s. (A and B) In the presence of boiled gD; (C and D) in the presence of intact gD; (A and C) with 5 mM KCl; and (B and D) with 50 mM KCl. The results shown are representative of three independent experiments. Bar, 30 μm.

tin-3 signals colocalized with PSD-95 (unpublished data). The puncta, which were immunoreactive for nectin-1 and synaptophysin but not for nectin-3, likely correspond to vesicle-filled axonal varicosities (Fig. 5 A, arrows in lower left insets), but it is not known whether these puncta correspond to cell–cell contact sites. Addition of gD resulted in ~16% decrease in size and concomitant 109% increase in number of the puncta immunoreactive for nectin-1, -3, and synaptophysin (Fig. 6 A). Similar results were obtained with the puncta immunoreactive for PSD-95 (unpublished data). After 16 d in culture, ~81% of the nectin-1–positive puncta were immunoreactive for synaptophysin (Fig. 5 B). Nectin-3 colocalized at ~74% of the puncta immunoreactive for both nectin-1 and synaptophysin. It is likely that nectin-1 and synaptophysin localize at the vesicle-filled presynaptic side and nectin-3 localizes at the postsynaptic side. Addition of gD resulted in ~35% decrease in size and concomitant 149% increase in number of the puncta immunoreactive for nectin-1, -3, and synaptophysin (Fig. 6 B). These effects of gD were dose and time dependent. Similar results were obtained with the puncta immunoreactive for PSD-95 (unpublished data). Boiled gD (100°C for 10 min) had no significant effect on the formation of synapses at any concentrations tested in primary cultured hippocampal neurons (unpublished data). The effects of gD on the formation of synapses were not due to its cell toxicity, because the neurons treated with gD showed vesicle recycling as active as the control neurons, as estimated by measuring vesicle recycling in response to high K^+ stimulation using a fluorescent dye (Fig. 7, A–D). These results indicate that gD induces a decrease in size and a concomitant increase in number of synapses.

Discussion

Asymmetric localization of nectin-1 and -3 at puncta adherentia junctions

We have previously shown that each member of the nectin family forms a homo-cis-dimer, followed by formation of a homo-trans-dimer (Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Sakisaka et al., 2001). Nectin-3 furthermore forms a hetero-trans-dimer with either nectin-1 or -2, and the formation of each hetero-trans-dimer is stronger than that of each homo-trans-dimer (Satoh-Horikawa et al., 2000). Nectin-4 also forms a hetero-trans-dimer with nectin-1, and the formation of the hetero-trans-dimer is stronger than that of the homo-trans-dimer (Reymond et al., 2001). However, it remains unknown whether the hetero-trans-dimer is indeed formed in vivo. We have shown here that nectin-1 and -3 localize asymmetrically at puncta adherentia junctions of the synapses between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area of adult mouse hippocampus. The localization patterns of nectin-1 and -3 strongly suggest that these nectins form a hetero-trans-dimer at puncta adherentia junctions. Puncta adherentia junctions, which are classified into a type of epithelial adherens junctions, have been regarded as symmetrical, based on the homo-trans-dimer of cadherin (Fannon and Colman, 1996). Therefore, our present findings indicate, for the first time, that in this region, the molecular architecture of puncta adherentia junctions is at least partially asymmetrical.

Our findings also shed new insight into the relationship between puncta adherentia and synaptic junctions. Although the relationship between the two types of junctions

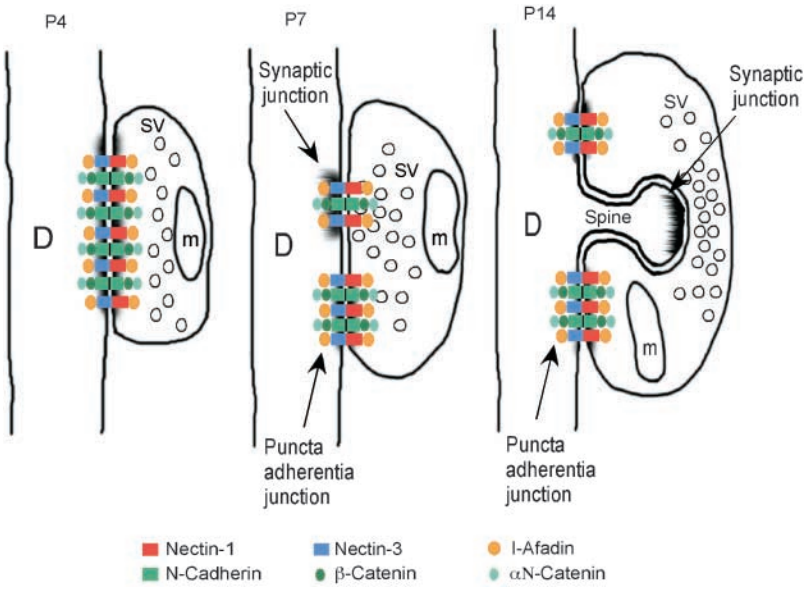


Figure 8. **Nectin–afadin and cadherin–catenin systems during the development of the synapses between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area.** The localization of the nectin–afadin system changes during the three developmental stages. The cadherin–catenin system is likely to colocalize with the nectin–afadin system at each stage, although we determined the localization of αN-catenin, but not that of N-cadherin or β-catenin, by immunoelectron microscopy. D, dendritic trunks of pyramidal cells; SV, synaptic vesicles; m, mitochondria.

in general is still controversial, their structural relationship at the synapses between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area has extensively been studied by Amaral and Dent (1981) (Fig. 8). They have found that this type of synapse is so highly developed that puncta adherentia and synaptic junctions must have their own functions. Six puncta adherentia and eight synaptic junctions on average are present in a single nerve terminal. The former junctions locate almost exclusively at cell–cell contact sites between the mossy fiber terminals and dendritic trunks of pyramidal cells, and the latter junctions are exclusively at the contact sites between synaptic vesicle–containing active zones of the mossy fiber terminals and the heads of highly lobulated dendritic spines. In the mossy fiber terminals, the cytoplasmic face of puncta adherentia junctions are not covered with synaptic vesicles but with a row of mitochondria. Therefore, synaptic junctions are regarded as neurotransmitter release sites and puncta adherentia junctions as mechanical anchoring sites, and transformation from one type of junction to the other is unlikely, at least in this highly developed type of synapse. Our morphological results indicate that nectin-1 and -3 are specific for puncta adherentia junctions, at least between the mossy fiber terminals and dendrites of pyramidal cells in the CA3 area of adult mouse hippocampus.

A possible role of the nectin–afadin system in domain segregation of synaptic and puncta adherentia junctions

We have identified changes in the distribution of the nectin–afadin system as well as the cadherin–catenin system during development of synapses (Fig. 8). At least three developmental stages are recognized during the maturation of synapses. At the first developmental stage, P4, nectin-1, -3, l-afadin, and αN-catenin colocalize at cell–cell contact sites, which are probably the most primitive synapses where morphological differentiation between synaptic and puncta adherentia junctions is not clearly observed. Appearance of the nectin–afadin and cadherin–catenin systems precedes membrane domain segregation of synaptic and puncta adherentia junctions. It is noteworthy that at the most primitive synapses, nectin-1 and -3 localize in an asymmetrical manner at cell–cell contact sites, suggesting that nectin-1 and -3 function as a hetero-trans-dimer from the beginning of synaptogenesis. At the second stage, P7, when morphological differentiation between the two domains is complete, the nectin–afadin system localizes with αN-catenin both at synaptic and puncta adherentia junctions. At the final stage, P14, when synaptic junctions are formed exclusively on the spines that stem from dendrites, the nectin–afadin system, together with the cadherin–catenin system, localizes at puncta adherentia junctions. These results suggest that during the maturation of synapses, membrane domain specialization gradually proceeds, and both the nectin–afadin and cadherin–catenin systems may participate in domain segregation and spine formation.

This neural membrane domain specialization is apparently similar to that found in the formation of the junctional complex, which mainly consists of adherens and tight junctions in epithelial cells, with respect to the order of junctional protein localization patterns. During the initial stage of the formation junctional complex in epithelial cells, primordial spot-like junctions are first formed at the tips of cellular protrusions radiating from adjacent cells (Yonemura et al., 1995; Adams et al., 1998; Ando-Akatsuka et al., 1999; Vasioukhin et al., 2000). The cadherin–catenin and nectin–afadin systems colocalize at the primordial junctions where claudin is not concentrated (Asakura et al., 1999; unpublished data). Claudin is a key cell–cell adhesion molecule that forms tight junctions (Tsukita et al., 1999). As cellular polarization proceeds, claudin gradually accumulates at the spot-like junctions to form tight junctions, and the cadherin–catenin and nectin–afadin systems are sorted out from claudin to form adherens junctions.

A role of the nectin–afadin system in formation of synapses

We have provided here the evidence that the nectin–afadin system plays an important role in the formation of synapses in

cultured rat hippocampal neurons by the use of gD, an inhibitor of nectin-1 (Sakisaka et al., 2001). We have found that gD induces a decrease in synapse size and a concomitant increase in synapse number. The exact mechanism of the decrease in synapse size remains to be clarified, but nectin-1, -3, l-afadin, αN-catenin, and N-cadherin colocalize at immature synapses during development, indicating the presence of clusters of the nectin–afadin and cadherin–catenin systems at the contact sites. We have previously shown that the nectin–afadin system organizes adherens junctions cooperatively with the cadherin–catenin system in nonneuronal cells (Ikeda et al., 1999; Takahashi et al., 1999; Miyahara et al., 2000; Tachibana et al., 2000). Therefore, the decrease in synapse size may be due to the partial inhibition of the hetero-trans-dimer between nectin-1 and -3, which may affect the cadherin-mediated cell–cell adhesion and result in smaller synapses. The exact mechanism of the increase in synapse number in the presence of gD is also unknown, but may be due to either failure of determination of the proper positions of synapses or compensation for functionally less competent smaller synapses. Our present results indicate that the formation of the hetero-trans-dimer between nectin-1 and -3 plays an important role in determining the position and size of synapses.

It has been reported that inhibition of the N-cadherin function by its Abs results in the formation of synapses at the regions beyond the appropriate target cells in the chick retinotectal tract, suggesting that N-cadherin plays a role in the formation of synapses (Yamagata et al., 1995). Addition of anti–N- and anti–E-cadherin Abs and cadherin peptides has been shown to induce suppression of long-term potentiation in mouse hippocampus (Tang et al., 1998). Moreover, in mutant mice lacking cadherin-11, long-term potentiation in the CA1 area of mouse hippocampus is enhanced (Manabe et al., 2000). Our present results, together with the earlier observations, suggest that the nectin–afadin system, as well as the cadherin–catenin system, plays an important role in the formation of synapses. This role of the nectin–afadin system is consistent with the recent finding that nectin-1 is a gene responsible for cleft lip/palate–ectodermal dysplasia, which is characterized by mental retardation in addition to ectodermal dysplasia (Suzuki et al., 2000).

Materials and methods

Abs

Rabbit polyclonal anti–nectin-1, -2, and -3 Abs, which recognize the cytoplasmic domains of nectin-1α, -2α, and -3α, respectively, were prepared as previously described (Takahashi et al., 1999; Satoh-Horikawa et al., 2000; Sakisaka et al., 2001). Rat monoclonal anti–nectin-1, -2, and -3 Abs, which recognize the extracellular domains of nectin-1, -2, and -3, respectively, were also produced as previously described (Takahashi et al., 1999; Satoh-Horikawa et al., 2000). A rabbit polyclonal anti–l-afadin Ab, which specifically recognizes l-afadin, but not s-afadin, was made as described by Mandai et al. (1997). A rat monoclonal anti–αN-catenin Ab (NCAT5) (Uchida et al., 1996) was supplied by Dr. M. Takeichi (Kyoto University, Kyoto, Japan). Mouse monoclonal anti-synaptophysin and anti–N-cadherin Abs were purchased from Chemicon and Transduction Laboratories, respectively. Rabbit polyclonal anti–*N*-methyl-D-aspartate receptor and mouse monoclonal anti–PSD-95 Abs were from Chemicon and Affinity Bioreagents, Inc., respectively.

Immunofluorescence microscopy

Adult mice (ddy strain) were deeply anesthetized by ether, and perfused with freshly prepared 2% paraformaldehyde in PBS for 15 min. Brains were dissected out and cut with a razor into several coronal sections (~2-mm thickness), which were soaked with the same fixative at 4°C for 2 h. For cryoprotection, sections were placed into 20% sucrose solution for 2 h and 25% sucrose solution overnight. The sections were frozen using liquid nitrogen. Serial 10-μm-thick sections were cut in a cryostat. The samples were incubated with the primary Abs described above, followed by incubation with a secondary Ab (Amersham-Pharmacia Biotech) coupled with fluorescein, Texas red, or Cy5. After being washed with PBS, they were embedded and viewed with a confocal imaging system (BioRad Laboratories; MRC-1024).

Immunoelectron microscopy

Immunoelectron microscopy, using the silver-enhanced immunogold method, was performed as previously described (Mizoguchi et al., 1994). The 10-μm-thick sections were incubated with the primary Abs described above, followed by incubation with a secondary Ab coupled with 1.4-nm gold particles (Nanoprobes Inc.). The sample-bound gold particles were silver-enhanced by the HQ-silver kit (Nanoprobes Inc.) at 18°C for 12 min. The samples were again washed and postfixed with 0.5% osmium oxide in a buffer containing 100 mM cacodylate buffer, pH 7.3. They were dehydrated by passage through a graded series of ethanol (50, 70, 90, and 100%) and propylene oxide, and embedded in epoxy resin. From this sample, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then observed with an electron microscope (JEM-1200EX; JEOL).

Preparation of gD

gD, an envelop protein of herpes simplex virus type 1, an α-herpes virus, was prepared as previously described (Sakisaka et al., 2001). In brief, a baculovirus transfer vector for gD (285t) (amino acids 1–285) (Rux et al., 1998) was constructed as follows: pFastBac1-Msp-Fc was first constructed by subcloning the inserts encoding the honeybee melittin signal peptide (Tessier et al., 1991) and the human IgG Fc into pFastBac1 (GIBCO BRL). A cDNA fragment of gD (285t) was then inserted into pFastBac1-Msp-Fc to express the chimeric protein fused with the NH_2-terminal signal peptide and the COOH-terminal IgG Fc. A baculovirus bearing this cDNA was prepared according to the manufacturer's protocol. High Five insect cells (Invitrogen) were grown in serum-free medium EX-CELL 400 (JRH Biosciences), infected with the baculovirus, and cultured at 26°C for 72 h. The culture supernatant was collected and applied to a protein A–Sepharose column (Amersham Pharmacia Biotech.), and then eluted with 20 mM glycine buffer, pH 2.5. The eluted protein was immediately neutralized with 1 M Tris and dialyzed against PBS. The chimeric protein of gD (285t) was used as gD in this paper.

Primary culture of rat hippocampal neurons

Rat hippocampal neurons were cultured as previously described (Takeuchi et al., 1997). In brief, hippocampi were isolated from rat embryos (20-d gestation), dissociated, plated on poly-L-lysine–coated glass coverslips, and cultured in MEM with 10% horse serum. After 4 d in culture, the medium was replaced with MEM supplemented with N2 supplement, 1 mg/ml of ovalbumin, 1 mM pyruvate, and 5 mM cytosine arabinoside in the presence of various concentrations (0.06, 0.6, 3.0, 8.75, 35, and 70 μg/ml) of intact gD or gD boiled at 100°C for 10 min. After 10 d in culture, the neurons were fixed with 2% paraformaldehyde in PBS for 4 h. Alternatively, the medium was again replaced with a fresh medium in the presence of the same concentration of gD or boiled gD as added after 4 d in culture. The neurons were cultured for another 6 d and fixed with 2% paraformaldehyde in PBS for 4 h. The neurons were subjected to immunofluorescence microscopic analysis with the rabbit polyclonal anti–nectin-1, rat monoclonal anti–nectin-3, mouse monoclonal anti-synaptophysin, and mouse monoclonal anti–PSD-95 Abs. By the confocal imaging system, puncta immunoreactive for nectin-1, -3, and synaptophysin were counted as synapses, and their sizes were measured. At least 1,000 puncta per culture were measured. Puncta immunoreactive for PSD-95 were also counted and their sizes were measured. This quantitative analysis was performed using the public domain NIH image program v1.61.

Labeling with FM1-43

To determine the viability of cultured rat hippocampal neurons, the neurons were loaded with FM1-43 (Molecular Probes) by the addition of 50 mM KCl as previously described (Becherer et al., 2000; Schikorski and Stevens, 2001). In brief, after 16 d in culture, the neurons were incubated with the medium containing 50 mM KCl and 20 μM FM1-43 for 90 s. After being washed with HBSS, the neurons were incubated with HBSS at 37°C for 15 min. They were fixed with 4% paraformaldehyde in PBS at 37°C for 15 min. After being washed with PBS, the neurons were examined with the confocal imaging system.

Other procedures

Protein concentrations were determined with BSA as a reference protein (Bradford, 1976). SDS-PAGE was performed as previously described (Laemmli, 1970).

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