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Linkage of N-cadherin to multiple cytoskeletal elements revealed by a proteomic approach in hippocampal neurons

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ABSTRACT

The CNS synapse is an adhesive junction differentiated for chemical neurotransmission and is equipped with presynaptic vesicles and postsynaptic neurotransmitter receptors. Cell adhesion molecule cadherins not only maintain connections between pre- and postsynaptic membranes but also modulate the efficacy of synaptic transmission. Although the components of the cadherin-mediated adhesive apparatus have been studied extensively in various cell systems, the complete picture of these components, particularly at the synaptic junction, remains elusive. Here, we describe the proteomic assortment of the N-cadherin-mediated synaptic adhesion apparatus in cultured hippocampal neurons. N-cadherin immunoprecipitated from Triton X-100-solubilized neuronal extract contained equal amounts of β- and α-catenins, as well as F-actin-related membrane anchor proteins such as integrins bridged with α-actinin-4, and Na^+/K^+-ATPase bridged with spectrins. A close relative of β-catenin, plakoglobin, and its binding partner, desmoplakin, were also found, suggesting that a subset of the N-cadherin-mediated adhesive apparatus also anchors intermediate filaments. Moreover, dynein heavy chain and LEK1/CENPF/mitosin were found. This suggests that internalized pools of N-cadherin in trafficking vesicles are conveyed by dynein motors on microtubules. In addition, ARVCF and NPRAP/neurojungin/δ2-catenin, but not p120ctn/δ1-catenin or plakophilins-1, -2, -3, -4 (p0071), were found, suggesting other possible bridges to microtubules. Finally, synaptic stimulation by membrane depolarization resulted in an increased 93-kDa band, which corresponded to proteolytically truncated β-catenin. The integration of three different classes of cytoskeletal systems found in the synaptic N-cadherin complex may imply a dynamic switching of adhesive scaffolds in response to synaptic activity.

1. Introduction

The CNS synapse is an adhesive junction differentiated for chemical transmission and is equipped with presynaptic vesicles and postsynaptic neurotransmitter receptors. Cell adhesion molecules that link pre- and postsynaptic membranes not only maintain specific synaptic connections but also regulate the efficacy of synaptic transmission and plasticity (Bozdagi et al., 2004; Jungling et al., 2006; Okamura et al., 2004; Tanaka et al., 2000). Proteins assembled for the cadherin-mediated adherens junction have been extensively studied in various cell systems. However, different cadherins have been studied in distinct cell system models. Hence, it is unclear which proteins are genuinely assembled in synaptic junctions.

Cadherins are large superfamily of transmembrane cell adhesion proteins with characteristic extracellular repeats of about 110 amino acids. The cadherin superfamily comprises the classic cadherin and protocadherin subfamilies. Classic cadherins share conserved cytoplasmic domains that bind to β-catenin, whereas protocadherins have diversiform cytoplasmic domains connected to a wide variety of intracellular signaling molecules (Yagi and Takeichi, 2000). Classic cadherins are expressed in synaptic junctions (Benson and Tanaka, 1998; Fannon and Colman, 1996; Manabe et al., 2000; Uchida et al., 1996). Among them, N-cadherin

Abbreviations: AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; CNS, central nervous system; HBSS, Hanks' balanced salt solution; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MAP kinase, mitogen-activated protein kinase; MT, microtubule; NMDA, *N*-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; PPW, presynaptic particle web; PSD, postsynaptic density; SDS, sodium dodecyl sulfate.

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has been intensively investigated in hippocampal neurons and has been proven to play various roles in synaptic transmission and plasticity (Bozdagi et al., 2000; Jungling et al., 2006; Tang et al., 1998). The involvement of N-cadherin in synaptic activity is also supported by studies on cadherin-interacting proteins such as β-catenin (Okuda et al., 2007), δ-catenin (Israely et al., 2004), and IQ-GAP/ERK (Schrick et al., 2007).

Accumulating evidence indicates the involvement of N-cadherin as a dynamic feature of synaptic physiology. For example, N-cadherin mediates an activity-induced enlargement of the dendritic spine, which is dependent on rearrangement of the actin cytoskeleton (Okamura et al., 2004). Concomitantly, N-cadherin can undergo conformational change, e.g. dimerization, with acquired resistance to trypsin (Tanaka et al., 2000). At the same time, N-cadherin shows enhanced binding to β-catenin (Murase et al., 2002) and stabilization on the cell surface (Tai et al., 2007). The immobilization of N-cadherin/β-catenin parallels with the stabilization of actin filaments by synaptic stimulation (Fischer et al., 2000; Fukazawa et al., 2003; Lin et al., 2005; Okamoto et al., 2004; Star et al., 2002). There are also suggestions that the N-cadherin–catenin complex is more directly linked to presynaptic vesicular physiology (Bamji et al., 2003, 2006; Bozdagi et al., 2004; Jungling et al., 2006) as well as postsynaptic neurotransmitter receptors (Saglietti et al., 2007). Furthermore, there are pathways that force N-cadherin into intracellular compartments. One pathway involves steady-state endocytosis, which is inhibited by the activation of the NMDA receptor (Tai et al., 2007). Another is an activity-regulated endocytic pathway, in which arcadlin (protocadherin-8), induces endocytosis of N-cadherin as a downstream event of p38 MAP kinase activation (Yasuda et al., 2007).

Thus, N-cadherin plays multiple, dynamic roles in synaptic remodeling and plasticity. Synaptic N-cadherin should therefore be linked to distinct cellular mechanisms that are specialized for separate, specific tasks. Here, we focus on the rodent hippocampal synapse to directly depict the cadherin-mediated adhesion complex. We describe the proteomic assortment of the synaptic adhesion apparatus assisted by N-cadherin in cultured hippocampal neurons. The protein complexes assembled with N-cadherin were consistent with the N-cadherin linkages to F-actins, intermediate filaments, and microtubules. In addition, synaptic stimulation by membrane depolarization resulted in the increase of a 93-kDa band, which corresponded to proteolytically truncated β-catenin. The integration of three different cytoskeletal systems takes place in N-cadherin-mediated adhesive machinery, providing a dynamic scaffold underlying synaptic plasticity.

2. Materials and methods

2.1. Neuron culture

Hippocampal neurons were cultured from E18 rat embryos as previously described (Tanaka et al., 2000). Neurons were plated at a density of 1.4–2.1 × 10^4 cells/cm^2 onto poly-L-lysine-coated dishes (Φ = 35 mm). Neurons were maintained in Neurobasal medium supplemented with B27 (Life Technologies) and 5 μM cytosine arabinoside.

2.2. Protein extraction, immunoprecipitation, and immunoblotting

Hippocampi were dissected from adult C57BL/6 mice, homogenized in Ca-PI-lysis buffer (10 mM HEPES–NaOH (pH 7.4), 1% Triton X-100, 120 mM NaCl, 2 mM $CaCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 20 mM β-glycerophosphate, and 1 mM Na_3VO_4) with a Teflon-glass homogenizer, and centrifuged for 1 h to obtain clear protein extracts. Mature neurons cultured for 4–5 weeks were depolarized with 50 mM KCl for 15 min. Neurons were washed twice with chilled PBS, harvested with 80 μl (for a Φ = 35 mm dish) of Ca-PI-lysis buffer, sonicated, and centrifuged. N-cadherin-associated protein complex was immunoprecipitated from these samples with 0.5–2.5 μg of murine anti-N-cadherin (BD Transduction Laboratories). The precipitated protein complex was separated in 4–15% gradient SDS–PAGE followed by either silver staining, Sypro Ruby (Molecular Probes) staining, or immunoblotting. Immunoblots were probed with murine anti-α-catenin (BD Transduction Laboratories, 1:5000), murine anti-p120 (BD Transduction Laboratories, 1:2500), murine anti-plakoglobin (BD Transduction Laboratories, 1:2000), rabbit anti-syntaxin 1 (Sigma–Aldrich, 1:10000), rabbit anti-JLP (SPAG9) (Abcam, 1:10000), rabbit anti-pan-14-3-3 (Millipore, 1:5000), murine anti-TRAP1 (BD Transduction Laboratories 1:5000), rabbit anti-DDB1 (Abcam, 1:5000), and rabbit anti-TRIM33 (Bethyl Laboratories, 1:2000) antibodies.

2.3. Sequential extraction of synaptosome fractions

Hippocampi isolated from two mice were homogenized (Dounce homogenizer, 30 strokes) in buffered sucrose (0.32 M sucrose in hypotonic A buffer). Hypotonic A buffer contained 4 mM HEPES–NaOH (pH 7.4), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.0025% butylated hydroxy toluene. The homogenate was centrifuged (800g × 10 min) and the supernatant (S1) was centrifuged again (9200g × 15 min) to collect the precipitate (P2). The P2 pellet was resuspended in buffered sucrose and overlaid on stepwise sucrose gradients (0.85, 1.0, and 1.2 M) and ultracentrifuged at 82,500g for 120 min. The crude synaptosome fraction accumulated between 1.0 and 1.2 M cushions was collected, diluted with 3 × volume of hypotonic A buffer, and ultracentrifuged (32,800g × 20 min). The resultant synaptosome pellet was resuspended in 120 μl of isotonic buffer B (10 mM HEPES–NaOH (pH 7.4), 120 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.0025% butylated hydroxy toluene, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride), mixed with 30 μl of 0.1 M methyl-β-cyclodextrin, and incubated at 37 °C for 15 min followed by centrifugation (15,000 rpm × 15 min at 23 °C). After removing the supernatant as a methyl-β-cyclodextrin extract, the pellet was resuspended in 150 μl of stripping buffer (5 M urea dissolved in isotonic buffer B) and centrifuged as previously to obtain a urea extract and stripped-membrane pellet. The urea-stripped membrane pellet was resuspended in 150 μl of solubilizing buffer (1% Triton X-100 dissolved in isotonic buffer B) and centrifuged to obtain a Triton extract and an insoluble pellet.

2.4. In-gel digestion

Gel pieces were excised from Sypro Ruby-stained gels and washed with 50% (v/v) acetonitrile and 50 mM NH_4HCO_3, pH 8.0, for 30 min to remove the fluorescence dye. Gel pieces were then soaked in acetonitrile for 5 min, acetonitrile was removed, and the gel pieces were dried for 20 min in a vacuum. Prior to enzymatic digestion, gel pieces were reduced with 10 mM dithiothreitol in 50 mM NH_4HCO_3 at 37 °C for 30 min, then alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min, and dehydrated by addition of acetonitrile. The reduced and alkylated gel pieces were rehydrated in 50 mM Tris–HCl, pH 9.0, and 0.5 μg/ml sequencing grade modified trypsin (Roche Diagnostics). Once this solution was fully absorbed by the gel pieces, enzyme-free Tris–HCl buffer was added until the gel pieces were covered. The samples were digested for 16 h at 37 °C, extracted with acetonitrile and 5% formic acid for 20 min, and acetonitrile was evaporated using a Speed-Vac centrifuge. The tryptic digests were desalted