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Nedd4 is a specific E3 ubiquitin ligase for the NMDA receptor subunit GluN2D

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ABSTRACT

NMDA receptors are a family of glutamate-gated ion channels that regulate various CNS functions such as synaptic plasticity and learning. However hypo-or hyper-activation of NMDA receptors is critically involved in many neurological and psychiatric conditions such as pain, stroke, epilepsy, neurodegeneration, schizophrenia, and depression. Thus, it is important to identify mechanisms (such as by targeted ubiquitination) that regulate the levels of individual subtypes of NMDA receptors. In this study, we used a series of tagged, carboxy terminal constructs of GluN2D to identify associating proteins from rat brain. Of seven different GluN2D C-terminal fragments used as bait, only the construct containing amino acids 983–1097 associated with an E3 ligase, Nedd4. A direct interaction between GluN2D and Nedd4 was confirmed both *in vivo* and *in vitro*. This association is mediated by an interaction between GluN2D's C-terminal PPXY motif and the 2nd and 3rd WW domains of Nedd4. Of the four GluN2 subunits, Nedd4 directly interacted with GluN2D and also weakly with GluN2A. Nedd4 coexpression with GluN2D enhances GluN2D ubiquitination and reduces GluN1/GluN2D NMDA receptor responses. These results identify Nedd4 as a novel binding partner for GluN2D and suggest a mechanism for the regulation of NMDA receptors that contains GluN2D subunit through ubiquitination-dependent downregulation.

This article is part of a Special Issue entitled 'GluR-dep synaptic plasticity'.

1. Introduction

NMDA receptors belong to the superfamily of ionotropic glutamate receptors and are well recognized for their essential role in various forms of synaptic plasticity and learning and memory (Watkins and Evans, 1981; Traynelis et al., 2010). NMDA receptor activation is critical for initiating long-term potentiation and long-term depression (Collingridge, 1987; Malenka and Bear, 2004) and experience-dependent synapse formation/elimination during development (Smith et al., 2009), as well as modulating neuroprotection and excitotoxicity (Hardingham, 2006).

NMDA receptors are heterotetrameric complexes of subunits from three classes: GluN1, GluN2 and GluN3 with most NMDA receptors being composed of two GluN1 and two GluN2 subunits (Traynelis et al., 2010). There are four subtypes of GluN2 subunits, GluN2A-GluN2D. GluN2D-containing receptors differ significantly from other NMDA receptors in their deactivation times (Misra et al., 2000), L-glutamate and glycine affinity (Ikeda et al., 1992; Buller et al., 1995), open probability (Traynelis et al., 2010), CNS distribution, and Mg^{++} sensitivity (Monyer et al., 1994). They are also developmentally down regulated with protein expression levels reduced by approximately 50–60% by the time of synaptogenesis (Wenzel et al., 1996) with an even greater degree of mRNA downregulation at this time (Watanabe et al., 1992; Wenzel et al., 1996). Hence NMDA receptors containing the GluN2D subunit are likely to have a specific role in NMDA receptor-dependent synapse formation/elimination that occurs during development and have additional roles in the adult brain.

GluN2D-containing NMDA receptors may also have a special role in neuropathological conditions. Tissue plasminogen activator (TPA) – enhanced stroke damage in the cerebral cortex has been found to be dependent specifically upon GluN2D subunits (Baron et al., 2010; Jullienne et al., 2011). GluN2D may also contribute to

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white matter injury (Micu et al., 2006) and Creutzfeldt-Jakob disease (Khosravani et al., 2008). Thus, it is important to identify systems that selectively regulate GluN2D containing NMDA receptor subpopulations.

In an effort to determine such mechanisms that regulate GluN2D subunits, we used a proteomic approach to identify proteins that associate with the GluN2D intracellular C-terminal tail. Although the four GluN2 sequences display high homology overall, the intracellular GluN2D C-terminal sequence displays very little homology to other glutamate receptor subunits (Ikeda et al., 1992). This sequence of 460 amino acids is highly enriched in proline residues in patterns reflecting a variety of potential protein–protein interaction motifs. These domains are likely to associate with proteins for downstream signaling of NMDA receptor activity as well as to recruit proteins for the regulation of NMDA receptor activity or degradation.

One such regulatory mechanism is the selective ubiquitination of specific proteins by individual members of the large family of E3-ubiquitin ligases (Yi and Ehlers, 2007). This provides a mechanism for targeting selected proteins for intracellular trafficking and degradation by the ubiquitin-proteosomal system (UPS), the lysosome, or the autophagosome (Clague and Urbe, 2010). In this report we identify and characterize the interaction between the E3 ligase Nedd4 (Neural precursor cell-expressed developmentally down-regulated) (Rotin and Kumar, 2009) and the GluN2D subunit. Nedd4 belongs to the HECT domain-containing family of E-3 ubiquitin ligase proteins (Rotin and Kumar, 2009). As the name reflects, Nedd4 was initially identified as a developmentally down-regulated gene from mouse neural precursor cells isolated from the neural tube (Kumar et al., 1992). Nedd4 is a 105 kDa protein with an N-terminal C2 domain, three WW domains (WW1, WW2 and WW3) in the middle region and a C-terminal with a ~ 350 amino acid long HECT domain (Fig. 1E) (Kumar et al., 1997).

2. Materials and methods

2.1. Antibodies and reagents

Affinity-purified mouse monoclonal antibody against epitope tag HA1.1 was purchased from Covance (Madison, WI). Affinity-purified rabbit antibody against green fluorescent protein (GFP) was purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal and rabbit polyclonal antibodies directed against Myc epitope were purchased from Sigma (St. Louis, MO). A mouse monoclonal antibody against ubiquitin was from BD Bioscience (San Jose, CA). Secondary donkey anti-rabbit horseradish peroxide (HRP) and donkey-anti-mouse HRP were obtained from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). The Gal 4 yeast two-hybrid system was purchased from BD Bioscience (San Jose, CA). The YPD agar was obtained from Bio 101 (La Jolla, CA). Bacto agar and tryptone were from BD Bioscience (San Jose, CA). Glutathione-agarose was acquired from GE healthcare (Piscataway, NJ). The Silver Snap Stain Kit II was from Thermo Fisher Scientific (Rockford, IL). The quick-coupled transcription and translation kit was from Promega (Madison, WI). Protease inhibitor cocktail were obtained from Sigma (St. Louis, MO). MG-132 was from Calbiochem (San Diego, CA) and ^{35}S –methionine was purchased from MP Biomedicals.

2.2. Construction of recombinant cDNA constructs

A total of seven overlapping and non-overlapping fragments of GluN2D cytoplasmic region were constructed by PCR amplifying the desired region from GluN2D *pBS* (kindly provided by Dr. Peter Seeburg, Max Planck Institute, Heidelberg, Germany) and sub-cloned in frame with the coding sequence of GST-tag of pGEX4T-1 vector (obtained from GE Healthcare, Piscataway, NJ). The GluN2D C-terminal fragment constructs were designed to minimize cutting in regions where peptide residues show a greater number of intra-protein contacts (using the CONpro bioinformatics algorithm (Pollastri et al., 2001). In retrospect, this approach prevented cutting within the Nedd4 binding region.

For direct yeast two-hybrid evaluation of protein–protein interactions, the sequence encoding the complete cytoplasmic region of GluN2D (GluN2D-ct; amino acid 841–1302) was amplified by a PCR reaction from full-length GluN2D *pBS* and sub-cloned into the multiple cloning site (MCS) of pGBKT7 yeast expression vector in frame with GAL4 DNA-binding domain using the restriction enzyme sites *NdeI* and *EcoRI*. Full-length sequence of Nedd4 was PCR-amplified from mouse Nedd4 cDNA construct (Magnifico et al., 2003) (plasmid 11426 from Addgene, Boston, MA) and subcloned into the MCS region of pGADT7 GAL4 DNA-activation domain using the restriction enzyme sites *NdeI* and *EcoRI*. The Nedd4 construct in pGADT7 yeast vector was used as a template to generate point mutations by using the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA) to generate phenylalanine in place of trytophan in WW domains at position 167 (WW1–W167F), position 323 (WW2–W323F) and position 378 (WW3–W378F). The GluN2D construct in pGBKT7 yeast vector was also used as a template to generate GluN2D C-terminal region carrying mutations in the PPSY motif (PPSY into AASF). Truncated GluN2D C-terminal constructs were generated by inserting stop codons resulting in fragments GluN2D-ctΔ940 and GluN2D-ctΔ1296 using the Quick-change site-directed mutagenesis kit. The yeast two-hybrid vector controls (GAL4-AD SV40 Large T-antigen and GAL4-BD p53) were purchased from BD Bioscience (San Jose, CA).

For *in vitro* transcription-coupled translation synthesis, various GluN2s C-terminal constructs were made by amplifying the C-terminal nucleotide coding sequence from GluN2A, GluN2B, GluN2C and GluN2D *pBS* (kindly provided by Dr. Peter Seeburg, Max Planck Institute, Heidelberg, Germany) and subcloning them inframe into the multiple cloning site of pSPUTK vector. For mammalian expression cDNA constructs, GFP-Nedd4 was constructed by amplifying full-length sequence of Nedd4 from Nedd4 cDNA and subcloning it into pEGFP-C1 mammalian expression vector (BD Bioscience) using the restriction sites *HindIII* and *KpnI* so that the nucleotide sequence of Nedd4 comes inframe with the GFP nucleotide sequence. HA-tagged ubiquitin in a mammalian vector was obtained from Addgene (Boston, MA). GST-labeled Nedd4 full-length, Nedd4-NH_2 terminal, Nedd4 C-terminal, WW1, WW2 and WW3 were made by amplifying the corresponding nucleotide sequence from the Nedd4 cDNA template and subcloning the segment into pGEX4T-1 vector using the restriction enzymes *BamHI* and *EcoRI*. All constructs and mutations have been verified at University of Nebraska Medical Center Sequencing facility and are freely available upon request.

2.3. Glutathione S-transferase (GST) pull-down assay

Seven different GST-tagged fragments of the GluN2D cytoplasmic region were obtained as purified proteins by first transforming the pGEX4T-1 plasmid GluN2D C-terminal constructs into *E. coli* BL21 cells (Invitrogen, Carlsbad, CA) and then inducing expression by adding IPTG for 3 h at 22 °C with constant shaking at 300 rpm. Cells were lysed and the GST-tagged fragments were purified by using Glutathione resin. Equal amounts of each of the seven different GST-labeled GluN2D fragments of were used as a bait to pull-down proteins from 10 day-old as well as adult rat brain. In brief, six 10-day old rat pups and 6 adult rats were halothane anaesthetized and their brains quickly removed and submerged into ice-cold lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EGTA and 1% CHAPS detergent) supplemented with protease and phosphates inhibitor cocktails (Sigma). Brains were homogenized by a series of 10 strokes in a Potter-Elvehjem tissue homogenizer followed by 10 s of sonication at a setting of 5. After rotating at 4 °C 30 min, the lysate was cleared from insoluble material by centrifugation at 20,800 × *g* for 30 min. Half of the cleared brain lysate was subjected to detergent removal by performing dialysis at 4 °C overnight using the Slide-A-Lyser dialysis cassette (Pierce, Rockford, IL). Equal amounts of brain lysate with and without the presence of CHAPS detergent were independently incubated with the seven GST-tag GluN2D cytoplasmic fragments and the mixture was rotated at 4 °C for 2–4 h. The Glutathione resin was washed with the lysis buffer for 5 times and the bound proteins were eluted with 4 M $MgCl_2$.

2.4. One dimensional SDS gel-electrophoresis and silver staining

In order to remove the presence of high amount of salt from the elution buffer, two methods were followed. In the first method, commercially available Ziptip-C18 (Millipore) was used and the proteins bound to the C18 column were eluted by washing the tip in 2× SDS sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 0.2 M DTT and 0.02% bromophenol blue). In an alternate procedure, salt was removed by precipitating the eluted proteins using ammonium acetate/10% methanol. The methanol residue was removed and the precipitated proteins were lyophilized to dryness using a SpeedVac concentrator (Thermo Electron, San Jose, CA) then the eluted proteins were again solubilized by using 2× SDS sample buffer. The bound proteins present in the SDS sample buffer were subjected to one-dimensional SDS electrophoresis using the 4–20% gradient Tris–HCl gels (Bio-Rad, Hercules, CA).

Soon after the completion of gel-electrophoresis, silver staining was performed on the gels by using the Silver Snap Stain (Thermo Fisher Scientific, Rockford, IL) according to the manufacture instructions. Silver stained gels were visualized on a light box; the interested bands were excised by a sharp razor blade and subsequently subjected to destaining. Gel slices were kept at 4 °C in 10% methanol until further analyzed by mass spectrometry.

2.5. Nano-LC-ESI-Qq-TOF Tandem Mass Spectrometry analysis

The gel pieces were recovered from the methanol storage solution and resuspended in 25 mM ammonium bicarbonate. The pieces in the mixture were reduced

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