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Research report

Regulation of ErbB-4 endocytosis by neuregulin in GABAergic hippocampal interneurons

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Abstract

Neuregulin (NRG)/ErbB receptor signaling pathways have recently been implicated in the reversal of long-term potentiation at hippocampal glutamatergic synapses. Moreover, polymorphisms in NRG-1 and ErbB-4 genes have been linked to an increased risk for developing schizophrenia. ErbB-4 is highly expressed at glutamatergic synapses where it binds to PSD-95 via its carboxyl terminal T–V–V sequence. Here we investigated the expression, localization and trafficking of ErbB-4 in cultured hippocampal neurons by immunocytochemistry, surface protein biotinylation, and live labeling of native receptors. We show that neuronal ErbB-4 is detected at its highest levels in GABAergic interneurons, as observed *in vivo*. ErbB-4 immunoreactivity precedes PSD-95 expression, with ErbB-4 cluster initially forming in the absence of, but later associating with, PSD-95-positive puncta. By surface protein biotinylation, the fraction of ErbB-4 receptors on the plasma membrane increases from 30% to 65% between 6 and 16 days *in vitro* (DIV). Interestingly, 30 min of NRG stimulation triggers measurable ErbB-4 receptor internalization at DIV 16, despite increased colocalization with PSD-95. We also investigated the role of TNFα-converting enzyme (TACE)-mediated receptor processing in regulating ErbB-4 surface expression. We found that the cleavage-resistant JM-b isoform accounts for 80% of all ErbB-4 transcripts in cultured hippocampal neurons. Receptor stimulation or treatment with phorbol esters does not induce detectable ErbB-4 processing, indicating that neurons mostly rely on endocytosis of the intact receptor to regulate ErbB-4 surface expression. These results enhance our understanding of the regulation of ErbB-4—mediated signaling at glutamatergic synapses.
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1. Introduction

ErbB receptors, including the EGF receptor (ErbB-1), ErbB-2, ErbB-3 and ErbB-4, comprise a family of receptor tyrosine kinases involved in numerous signaling processes including the control of cell growth and differentiation. In particular, ErbB2-4 and their cognate neuregulin (NRG) ligands have long been recognized as critical mediators of cell fate, proliferation, migration and differentiation processes in the developing peripheral and central nervous system (for review, see [1,6,10,12,16]. Although NRGs and ErbB receptors continue to be expressed at high levels, until recently their functions in the adult brain were unknown. Work from our laboratory, as well as others, has implicated NRG/ErbB signaling in regulating plasticity of glutamatergic synapses. At CA3-to-CA1 synapses in the hippocampus, activation of ErbB receptor signaling can prevent or revert long-term potentiation (LTP) [21,23]. Depotentiation of LTP in hippocampal neurons is mediated by the internalization of GluR1-containing AMPA receptors without affecting NMDA receptor-evoked postsynaptic currents. Conversely, stimulation of NRG/ErbB signaling in the prefrontal cortex leads to altered surface expression of NMDA receptors at glutamatergic

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synapses on pyramidal neurons [18]. Consistent with its ability to regulate synaptic plasticity, there is a rapidly growing body of evidence supporting the involvement of the NRG/ErbB pathway, and interactions between ErbB-4 and PSD-95, in the pathogenesis underlying schizophrenia [9,19,20].

By *in situ* hybridization, ErbB2-4 receptor genes are expressed in the CA1-CA3 regions of the hippocampus, with highest ErbB-4 mRNA levels found in interneurons [17,24,25]. The distribution of ErbB-4 protein in different neuronal populations is less clear; nevertheless, there is general agreement that the highest receptor levels are in GABAergic neurons (see Section 4). ErbB-4 and NMDA receptors colocalize in postsynaptic densities (PSDs) at glutamatergic synapses. ErbB-4 physically interacts via its c-terminal T–V–V sequence with membrane-associated guanylate kinases (MAGUKs) such as PSD-95, SAP-102 and PSD-93 [14,21]. PSD-95 is a major scaffolding component of PSDs, and together with other MAGUKs, plays an important role in organizing the intricate network of receptors, signaling molecules and cytoskeletal adaptor proteins that together mediate synaptic transmission and plasticity (see, for example: [4,15,22]). The association with the PSD positions ErbB-4 as a potentially important modulator of synaptic plasticity, and further supports the notion that NRG/ErbB signaling regulates synaptic plasticity *in vivo*.

Many growth factor receptors are internalized upon ligand binding to gradually attenuate receptor signaling and to desensitize the cell to excess ligand availability, but also to target activated receptors to other intracellular substrates [37]. Unlike the EGF receptor (ErbB-1), rapid ligand-dependent receptor internalization is notably low for all other ErbB receptors in cancer cell lines expressing ErbB-2 or ErbB-3, or transfected NIH 3T3 cells overexpressing ErbB-4 [3]. Rather, ligand-mediated proteolytic processing has been proposed as a densitization mechanism for ErbB-4 [7,40]. Specifically, it was found that ligand-induced cleavage of the 120 kDa ectodomain by TNF-alpha converting enzyme (TACE; syn. Adam 17) serves to shed the receptor from the surface [31,35,36,40]. Sequence determinants for susceptibility to TACE-dependent ErbB-4 processing reside in a juxtamembrane region that is included in JM-a but that is missing from JM-b transcripts [11]. Interestingly, a recent study reported that JM-a isoforms were upregulated in postmortem brains from schizophrenic individuals [33].

Trafficking of ErbB-4 in neurons has been studied mainly with regard to its NRG-dependent recruitment to lipid rafts [27], a process which is believed to help targeting the ErbB-4 signaling complex to synapses [38]. However, to better understand the emerging role of ErbB receptors, in particular of ErbB-4, as modulators of synaptic plasticity in the adult brain, it is critical to know how receptor availability is regulated in neurons. As our present knowledge about endocytosis of ErbB receptors is largely based on cell lines lacking the functional specializations of mature neurons such as the postsynaptic density, it is unclear if, and how, ErbB-4 receptor processing and endocytosis are regulated in neurons in response to NRG binding. To address this question, we have investigated the surface expression and NRG-stimulated endocytosis of ErbB-4 in dissociated hippocampal neurons by surface protein biotinylation and live cell labeling of endogenous receptors, as well as the role of TACE-mediated ectodomain shedding in regulating surface ErbB-4.

2. Material and methods

2.1. Materials

Human NRG1β1 EGF domain peptide (amino acids 176–246; R&D systems (Minneapolis, MN)) was reconstituted at 5 μM in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). 4-[(3-bromophenyl)amino]-6-(methylamino)-pyrido[3,4-d]pyrimidine (PD158780), PMA (4α-Phorbol 12-myristate 13-acetate) and the TACE inhibitor TAPI-2 were from Calbiochem (La Jolla, CA). Mouse monoclonal antibodies ab77 and ab72 against the extracellular domain of ErbB-4 [8] and Ab-4 against tubulin were from Lab Vision Corporation (Fremont, CA). Rabbit polyclonal antibody C-18 against the carboxyl terminus of ErbB-4 was from Santa Cruz Biotechnologies (Santa Cruz, CA), mouse monoclonal antibody 7E3-1B8 against PSD-95 was from Affinity Bioreagents (Golden, CO), rabbit polyclonal antibody against GABA was from Sigma (St. Louis, MO), and mouse monoclonal antibody PY-20 against phosphotyrosine was from BD Transductions Laboratories (Lexington, KY). Secondary antibodies conjugated to horseradish peroxidase, Cyanine 3 (Cy3) and Alexa 488 were from Amersham (Piscataway, NJ), Jackson ImmunoResearch (West Grove, PA) and Molecular Probes (Eugene, OR), respectively. A plasmid expressing PSD-95 was a kind gift by D. Bredt.

2.2. Cell culture

For post-fixation immunofluorescence and the analysis of ErbB-4 JM-a/b isoform expression, dissociated hippocampal neurons essentially free of glia from embryonic day (E19) Sprague Dawley rats were maintained in serum-free neurobasal medium supplemented with B-27 (Invitrogen, San Diego, CA), as previously described [5,26]. For protein biochemistry experiments and antibody feeding of live neurons, cells were plated on a confluent glial monolayer, as previously described [14].

2.3. Semi-quantitative RT-PCR of ErbB-4 JM-a and JM-b splice variants

Total RNA was extracted from rat hippocampal neurons (DIV 6, 16, and 22) and from mouse whole brain using RNAwiz (Ambion, Austin, TX), and reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen). Primers for the amplification of the sequence encompassing the alternatively spliced exon encoding JM-a were: ErbB-4_JM5′ (rat: 5′-GAAATGTCCAGATGTCCTACAGGG-3′; mouse: 5′-GAAATGTCCAGATGGCCTACAGGG-3′) and ErbB-4_JM3′ (rat: 5′-CTTTTTGATGCTCTTCCTTCTGAC-3′; mouse: 5′-CTTTTTGATGCTCTTTCTTCTGAC-3′) [11]. Target sequences were amplified using Taq Expand (Roche, Indianapolis, IN) at 63 °C annealing temperature for 26, 28 and 30 cycles. Aliquots were separated on 2% agarose, transferred to nylon membrane and probed with a [γ-^{32}P]-labeled nested oligonucleotide (5′-AGC AAA CAG TTT CAT ATT TAA GTA CG-3′) against a sequence common to JM-a and JM-b—derived PCR products. Blots were scanned on a Storm 840 phosphorimager (Amersham) and signals were quantified using ImageQuant5.2 software.

2.4. Surface protein biotinylation of endogenous ErbB-4

Hippocampal neurons were washed 3 times with progressively colder PBS containing Ca^{2+} and Mg^{2+}. Surface proteins were labeled with 1 mg/ml NHS-SS-biotin (Pierce, Rockford, IL) in PBS at 4 °C for 20 min, and then washed three times with cold 25 mM glycine in PBS. Cells were harvested in 1 ml of lysis buffer (0.84% Triton X-100, 0.16% SDS in PB solution: 10 mM sodium phosphate, pH 7.4, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 50 mM NaF, and 10 μg/ml aprotinin) at 4 °C. The lysates were briefly sonicated, heated at 60 °C for 5 min and spun at 20,000 × *g* at 4 °C. Supernatants were incubated with 300 μl of streptavidin beads (UltraLink Plus; Pierce) at 4 °C for 2 h. Aliquots of the slurry were saved to estimate total