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Regulation of Neuregulin Signaling by PSD-95 Interacting with ErbB4 at CNS Synapses

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Summary

Neuregulins (NRGs) and their receptors, the ErbB protein tyrosine kinases, are essential for neuronal development, but their functions in the adult CNS are unknown. We report that ErbB4 is enriched in the postsynaptic density (PSD) and associates with PSD-95. Heterologous expression of PSD-95 enhanced NRG activation of ErbB4 and MAP kinase. Conversely, inhibiting expression of PSD-95 in neurons attenuated NRG-mediated activation of MAP kinase. PSD-95 formed a ternary complex with two molecules of ErbB4, suggesting that PSD-95 facilitates ErbB4 dimerization. Finally, NRG suppressed induction of long-term potentiation in the hippocampal CA1 region without affecting basal synaptic transmission. Thus, NRG signaling may be synaptic and regulated by PSD-95. A role of NRG signaling in the adult CNS may be modulation of synaptic plasticity.

Introduction

Efficient and accurate synaptic transmission is guaranteed by compartmentalization of both pre- and postsynaptic signaling molecules at the synapse. PSD-95 (also known as SAP90), a protein enriched in the PSD, appears to be important for molecular organization of the postsynaptic complex in neurons (Garner and Kindler, 1996; Kennedy, 1997; Sheng and Wyszynski, 1997; Craven and Bredt, 1998). It has three N-terminal PDZ domains, a src homology 3 (SH3) domain, and a guanylate-kinase-like domain in the C-terminal half of the protein. PDZ domains are ~80–90 amino acid motifs that were originally identified in PSD-95, the *Drosophila* septate junction protein discs large (DLG), and the epithelial tight-junction protein zona occludens 1 (ZO1) (Woods and Bryant, 1991; Cho et al., 1992; Kistner et al., 1993; Willott et al., 1993). PSD-95 interacts, via the PDZ domains, with the intracellular C termini of NMDA-type glutamate receptors (Kornau et al., 1995) and Shaker K^+ channels (Kim et al., 1995) and can cluster NMDA receptors (NMDARs) or K^+ channels when coexpressed in heterologous cells (Kim et al., 1996; Niethammer et al., 1996). In *Drosophila*, mutations of *dlg*, the *Drosophila* homolog of PSD-95, results in abnormal synaptic morphology (Lahey et al., 1994) and dislocation of Shaker K^+ channels (Tejedor et al., 1997). PSD-95 may be important in the localization of neurotransmitter receptors and ion channels at the postsynaptic membrane and in the formation of heterotypic intercellular junctions between different neurons (Irie et al., 1997).

NRGs are a family of structurally related polypeptides, each of which contains an epidermal growth factor (EGF)-like domain. They are encoded by four distinct genes (*nrg-1* to *nrg-4*) (Holmes et al., 1992; Peles et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Zhang et al., 1997). The structure and function of NRG-1 have been extensively characterized. NRG-1 was simultaneously identified as a neuronal factor essential for the proliferation and survival of Schwann cells (glia growth factor) (Marchionni et al., 1993), as a factor that stimulates the synthesis of muscle AChR (ARIA for acetylcholine receptor–inducing activity) (Falls et al., 1993), and as a ligand of the receptor tyrosine kinase ErbB2 (NDF for neu differentiation factor or heregulin) (Holmes et al., 1992; Peles et al., 1992; Wen et al., 1992). NRG-1 is produced in various types of neurons during development (Chen et al., 1994; Corfas et al., 1995) and has multiple functions essential for the development of the nervous system as well as the heart. It promotes growth and differentiation of Schwann cells and astrocytes (Shah et al., 1994) and can rescue Schwann cell precursors from apoptosis (Dong et al., 1995). NRG-1 appears to play a crucial role in the migration of cerebellar granule cells along radial glial fibers (Anton et al., 1997; Rio et al., 1997). During synaptogenesis, NRG-1 induces the expression of acetylcholine receptor at the neuromuscular junction (NMJ) (Falls et al., 1993; Si et al., 1996, 1999) and in CNS neurons (Yang et al., 1998) and of the NMDAR NR2C subunit in cerebellar granule cells (Ozaki et al., 1997). Mutation of the *nrg-1* gene result in impaired CNS development (Meyer and Birchmeier, 1995).

There are three ErbB proteins, ErbB2, ErbB3, and

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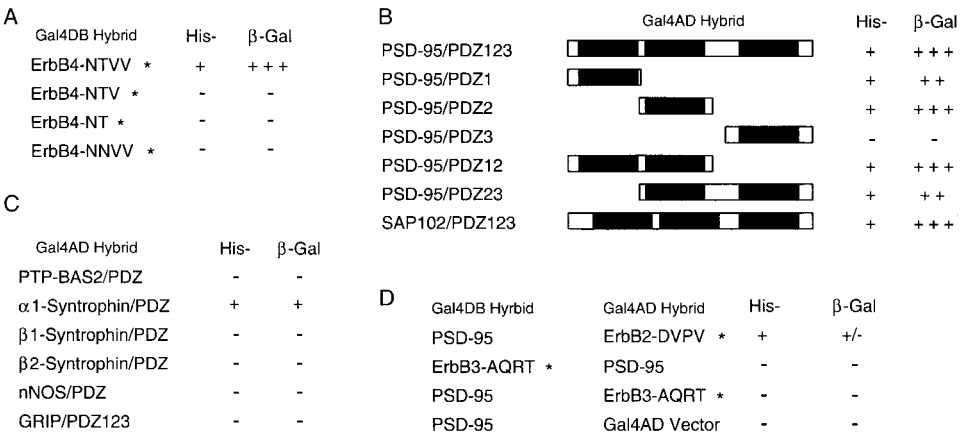

Figure 1. Binding of ErbB4 with PSD-95 in Yeast

(A) Dependence of the interaction between ErbB4 and PSD-95 on ErbB4 C terminus. Asterisks indicate amino acid residues prior to stop codon. Yeast cells were cotransformed with a vector encoding the Gal4 DB fused to different ErbB4 C-terminal constructs and Gal4 AD/PSD-95.

(B) Dependence of the interaction between ErbB4 and PSD-95 on PDZ domains. Yeast cells were cotransformed with a vector encoding the Gal4 AD fused to various PDZ domains of PSD-95 and Gal4DB/ErbB4-NTVV*.

(C) Specificity of ErbB4 interaction with PDZ domain–containing proteins. Yeast cells were cotransformed with a vector encoding the Gal4 AD fused to PDZ domains of indicated proteins and Gal4DB/ErbB4-NTVV*.

(D) Interaction between ErbB2 or ErbB3 with PSD-95. Yeast cells were cotransformed with a pair of indicated constructs. Transformed yeast cells were seeded in His- plates and scored for growth and for β-gal activity.

ErbB4, that are members of the EGF receptor family (Burden and Yarden, 1997; Gassmann and Lemke, 1997). Each ErbB protein has an extracellular ligand binding domain, a single transmembrane domain, a short intracellular juxtamembrane region, a tyrosine kinase domain, and a carboxy-terminal tail. NRG receptors differ in kinase activity and substrate selectivity (Burden and Yarden, 1997). Although NRG-1 (NDF and heregulin) was purified as a ligand for tyrosine kinase ErbB2 activation, it does not bind to ErbB2 (Plowman et al., 1993a; Carraway and Cantley, 1994; Sliwkowski et al., 1994; Karunagaran et al., 1995). On the other hand, the catalytic activity of ErbB3 is impaired in comparison to the other ErbB proteins (Wallasch et al., 1995). NRG-1 binds to ErbB3 (Sliwkowski et al., 1994) and the ligand binding induces allosteric changes that favor the formation of heterodimers between ErbB2 and ErbB3 and thus activates the tyrosine kinases (Burden and Yarden, 1997). In terms of ErbB4, tyrosine kinase activation is predominantly mediated by ligand-induced homodimerization (Plowman et al., 1993a; Graus-Porta et al., 1997). Upon NRG stimulation, tyrosine residues in the carboxyl termini of ErbB protein tyrosine kinases become phosphorylated and serve as docking sites for cytoplasmic signaling molecules such as Shc, Grb2, and the p85 subunit of phosphatidylinositol-3 (PI3) kinase (Jo et al., 1995; Tansey et al., 1996; Won et al., 1999). Subsequently, a plethora of signaling molecules are activated, including the extracellular signal-regulated kinase (ERK) (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997), c-Jun N-terminal kinase (JNK) (Si et al., 1999), and PI3 kinase (Tansey et al., 1996). Activation of ERK is essential for NRG-mediated expression of acetylcholine receptors at the NMJ (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997).

Although NRGs and ErbB proteins are highly expressed in the adult CNS, their function is unknown. A conspicuous structural feature of the ErbB proteins is their extended, intracellular carboxy-terminal sequences that are poorly conserved among the ErbBs. These sequences contain the major sites for tyrosine phosphorylation and recruitment of downstream signaling molecules. In addition, they may assemble a signal-transducing complex for the NRG signaling and/or anchor the ErbBs in a subcellular compartment. Identification of proteins interacting with these sequences may therefore provide insights into NRG signaling or its regulation. In this paper, we demonstrate that ErbB4 interacts with PSD-95 in yeast, in mammalian cells, and in CNS synaptosomes. ErbB4 is enriched in the PSD and colocalizes with PSD-95 in hippocampal neurons. We provide evidence that PSD-95 enhances NRG signaling, probably by forming a ternary complex with two molecules of ErbB4. Furthermore, we demonstrate that NRG inhibits induction of tetanus-induced long-term potentiation (LTP) in hippocampal slices.

Results

Identification of PSD-95 as an ErbB4 Interacting Protein by a Yeast Two-Hybrid Screen

To identify proteins that bind to ErbBs, we used the yeast two-hybrid system. A yeast two-hybrid system screen of mouse cDNA libraries (Lumeng et al., 1999) (5×10^6 clones) using the ErbB4 carboxyl terminus as bait led to the finding of PSD-95. The last three amino acid residues of the ErbB4 carboxyl terminus, -TVV* (Plowman et al., 1993b), fit the consensus motif (-T/SXV*, where X could be any amino acid residue) critical for binding to the PDZ domains (Kornau et al., 1995). To determine whether ErbB4 interacts PSD-95 via ErbB4 C terminus, we examined the interaction between PSD-95 and ErbB4 mutants with deletion or mutation in the carboxyl terminus. Deletion of the last amino acid

residue valine or mutation of the −3 position threonine to asparagine prevented ErbB4 from interacting with PSD-95 (Figure 1A), indicating that the ErbB4 C terminus is required for the interaction between ErbB4 and PSD-95 and suggesting that PSD-95 interacts with ErbB4 via PDZ domains. To determine which PDZ domain of PSD-95 binds to ErbB4, we generated a variety of constructs encoding an individual or a combination of PDZ domains fused with the upstream Gal4 activation domain. As shown in Figure 1B, ErbB4 interacted with the first and second PDZ domains, but not the third PDZ domain. In addition to PSD-95, ErbB4 also interacted with SAP102, another member of the PSD-95 family (Figure 1B).

We tested the specificity of the interaction between ErbB4 and PSD-95 in yeast. ErbB4 did not interact with PDZ domains of PTP-BAS2, β1- or β2-syntrophin, nNOS, or GRIP. However, ErbB4 did interact with the PDZ domain of α1-syntrophin (Figure 1C). ErbB2, whose C terminus ends with -VPV*, also interacted with PSD-95, but the affinity for ErbB2 was ~20-fold less than that for ErbB4 as determined in liquid β-galactosidase assays. However, no interaction was detected between the C terminus of ErbB3 and PSD-95 (Figure 1D). These results demonstrated that ErbB4, via its C terminus, interacts with the first and second PDZ domains of PSD-95.

Interaction of ErbB4 with PSD-95 in Mammalian Cells

To determine whether ErbB4 interacts with PSD-95 in mammalian cells, we expressed ErbB proteins with or without PSD-95 in HEK293 cells. Lysates of transfected cells were immunoprecipitated with specific antibodies against individual ErbB proteins and immunoblotted with antibodies against PSD-95. PSD-95 was detected in immunoprecipitates from cells coexpressing ErbB4 (Figure 2A), suggesting that ErbB4 associates with PSD-95 in vivo. PSD-95 was detectable in ErbB2 immunoprecipitates but at much lower levels than with ErbB4. Expression of PSD-95 was similar under both conditions, suggesting that PSD-95 associated only weakly with ErbB2 when coexpressed in HEK293 cells. In contrast, PSD-95 was not detected in the ErbB3 immunoprecipitates (Figure 2A). The interaction of ErbB4 with PSD-95 required the intact C terminus because the addition of a haemaglutinin (HA) epitope to the C terminus of ErbB4, which blocked the -TVV* motif, abolished the interaction (Figure 2B).

Enrichment of ErbB4 in the PSD and In Vivo Interaction of ErbB4 with PSD-95

Since PSD-95 is localized at postsynaptic membrane specializations (Cho et al., 1992), our finding of the interaction between ErbB4 and PSD-95 suggests that ErbB proteins may be localized at excitatory synapses in the CNS. To determine whether ErbBs are present in the PSD fraction from of the CNS, we performed subcellular fractionation studies. In rat CNS, ErbB proteins were detected as 180 kDa proteins exclusively associated with membranes (Figure 3A). As with PSD-95, the membrane-associated ErbB4 and ErbB2 further purified into the PSD fraction, in which it was resistant to extraction by Triton X-100 (Figure 3A). The degree of ErbB4 and

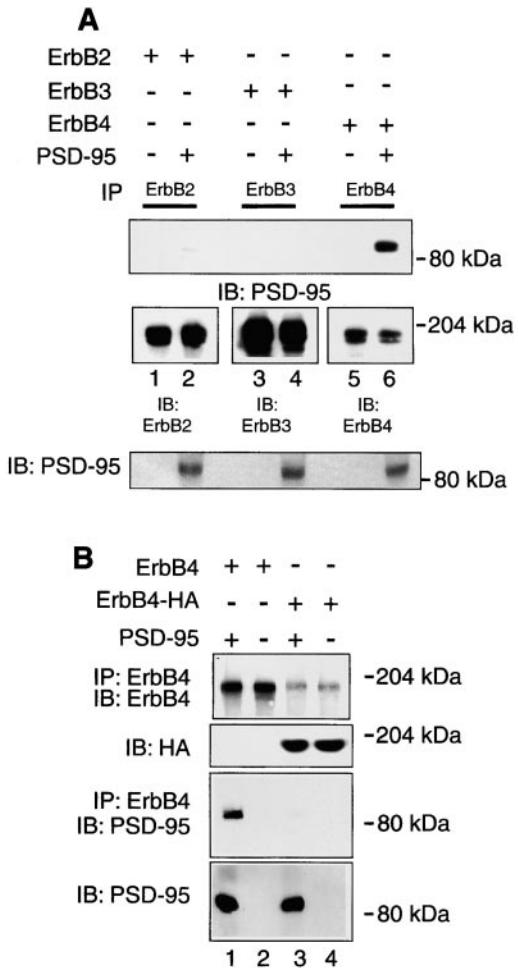

Figure 2. Interaction of ErbB4 with PSD-95 in Mammalian Cells

(A) Coimmunoprecipitation of ErbB4 and ErbB2 with PSD-95 in HEK293 cells. Cells transfected with indicated vectors were lysed. Immunoprecipitations (IP) with ErbB antibodies were revealed by immunoblotting (IB) with PSD-95 antibodies.

(B) ErbB4 with a blocked C terminus did not interact with PSD-95. HEK293 cells were transfected with ErbB4 or ErbB4-HA vectors with or without PSD-95. Immunoprecipitations with ErbB4 antibodies were revealed by immunoblotting with ErbB4 and PSD-95 antibodies, respectively. Expression of ErbB4-HA and PSD-95 was demonstrated by direct blotting cell lysates with HA and PSD-95 antibodies.

ErbB2 enrichment in the PSD fraction correlated strongly with that of PSD-95. ErbB3, however, was not detected in the PSD fraction although ErbB3 is a membrane-associated protein. These biochemical studies demonstrate that ErbB4 and ErbB2 are present in the PSD fraction and suggest that they are appropriately localized to form a protein complex in vivo with PSD-95.

To determine whether ErbB4 and ErbB2 interact with PSD-95 in the CNS, we investigated whether ErbB4 and ErbB2 could be coimmunoprecipitated with PSD-95 from rat brain. Synaptosomes of rat brain were solubilized with 1% deoxycholate and incubated with antibodies against ErbB proteins or PSD-95. As shown in Figure 3B (lanes 3 and 5), immunoprecipitation of ErbB4 or of ErbB2 resulted in coimmunoprecipitation of PSD-95. In contrast, PSD-95 was not detected in the ErbB3 immunoprecipitates. ErbB4 was not detected in reciprocal

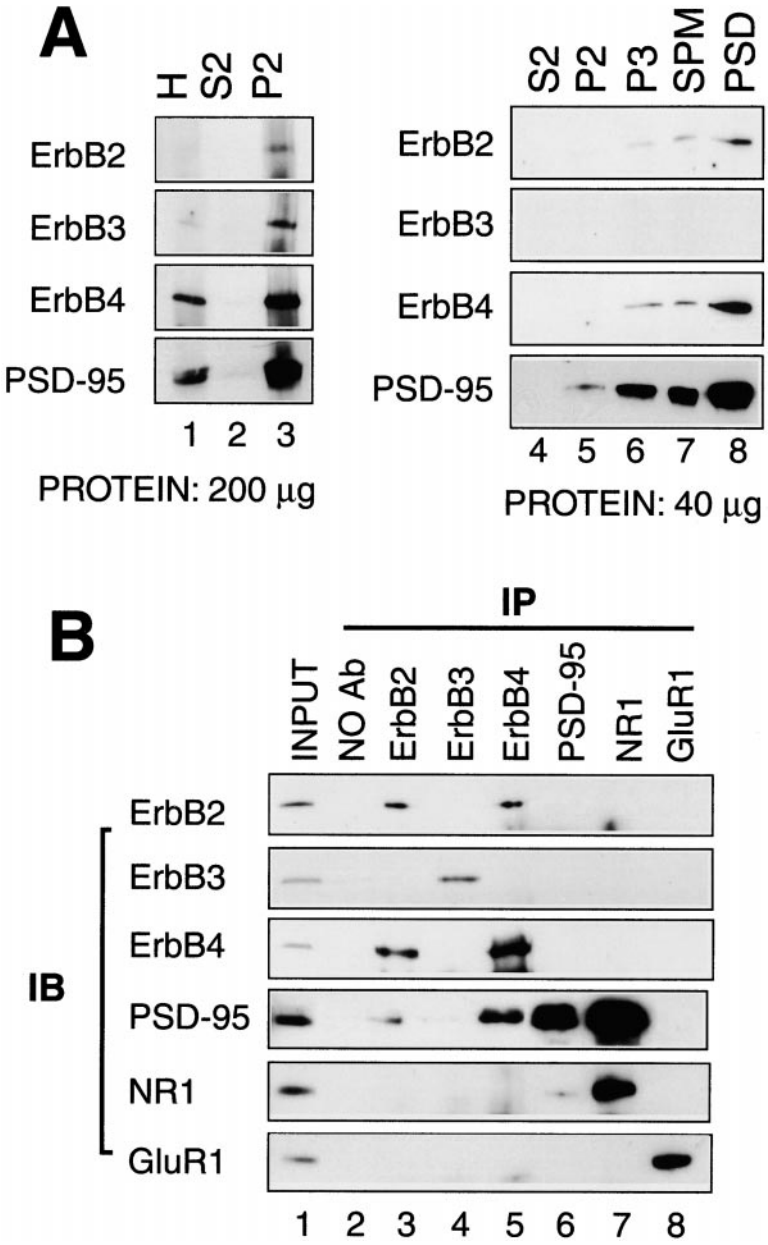

Figure 3. Enrichment of ErbB4 and ErbB2 in PSD and Interaction with PSD-95 in the CNS

(A) Enrichment of ErbB4 and ErbB2 in postsynaptic densities. Rat brain homogenates (H) were subjected to sequential centrifugations to yield cytosol (S2) and synaptosomes (P2). Washed synaptosomes (P3) were further fractionated by discontinuous sucrose gradient centrifugation to generate synaptosomal plasma membrane (SPM) that was treated with 0.4% Triton X-100. The insoluble SPM was designated as PSD. Samples were separated by SDS-PAGE, transferred to a nylon membrane, and immunoblotted with the respective antibodies.

(B) Interaction between ErbB4 and ErbB2 and PSD-95 in the CNS. Rat brain synaptosomes were solubilized with 1% deoxycholate, and the resulting detergent extract (INPUT) was used to immunoprecipitate (IP) the indicated proteins. The immunoprecipitates were then immunoblotted (IB) for ErbBs, PSD-95, NR1, and GluR1. "NO Ab" indicates precipitation reactions in the absence of antibodies. Three times of the amount of INPUT was used for immunoprecipitations.

coimmunoprecipitations with PSD-95 antibodies. A potential explanation for this finding is that ErbB4 may interact with a subpopulation of PSD-95, which is supported by results from immunocytochemical studies (see below). ErbB2 was also present in the ErbB4 immunoprecipitates (Figure 3B) and coenriched with ErbB4 and PSD-95 in the PSD fraction (Figure 3A). PSD-95 coimmunoprecipitated with NR1, the primary subunit of NMDARs (Figure 3B, lane 7). NR1 does not interact directly with PSD-95 but associates with NR2A or NR2B subunits that interact with PSD-95 via PDZ domains (Kornau et al., 1995). The nondenaturing immunoprecipitation condition did not interrupt the PSD-95/NR1/NR2 complex (Muller et al., 1996; Kim et al., 1997). As a negative control, we found that ErbB4 and ErbB2 were not present in immunoprecipitates of GluR1, a subunit of AMPA receptors (Figure 3B, lane 8).

Colocalization of ErbB4 with PSD-95 at Synapses of Hippocampal Neurons

We next studied the subcellular localization of ErbB proteins by double labeling hippocampal neurons with antibodies to ErbB4 or PSD-95. Neurons stained for ErbB4 showed very prominent clustered staining in neurites (Figure 4A). As reported previously (Kornau et al., 1995), PSD-95 staining was punctate in hippocampal neurons. Comparing the pattern of ErbB4 staining with that of PSD-95, we observed PSD-95 at all ErbB4 clusters, suggesting that ErbB4 is restricted to PSD-95 clusters. However, ErbB4 was not present in every PSD-95 cluster (Figure 4A), suggesting that ErbB4 interacts with only a subset of PSD-95 protein. Double staining of hippocampal neurons with antibodies against ErbB4 and the synaptic marker synaptophysin confirmed that ErbB4 is localized at synapses (Figure 4B). In agreement with

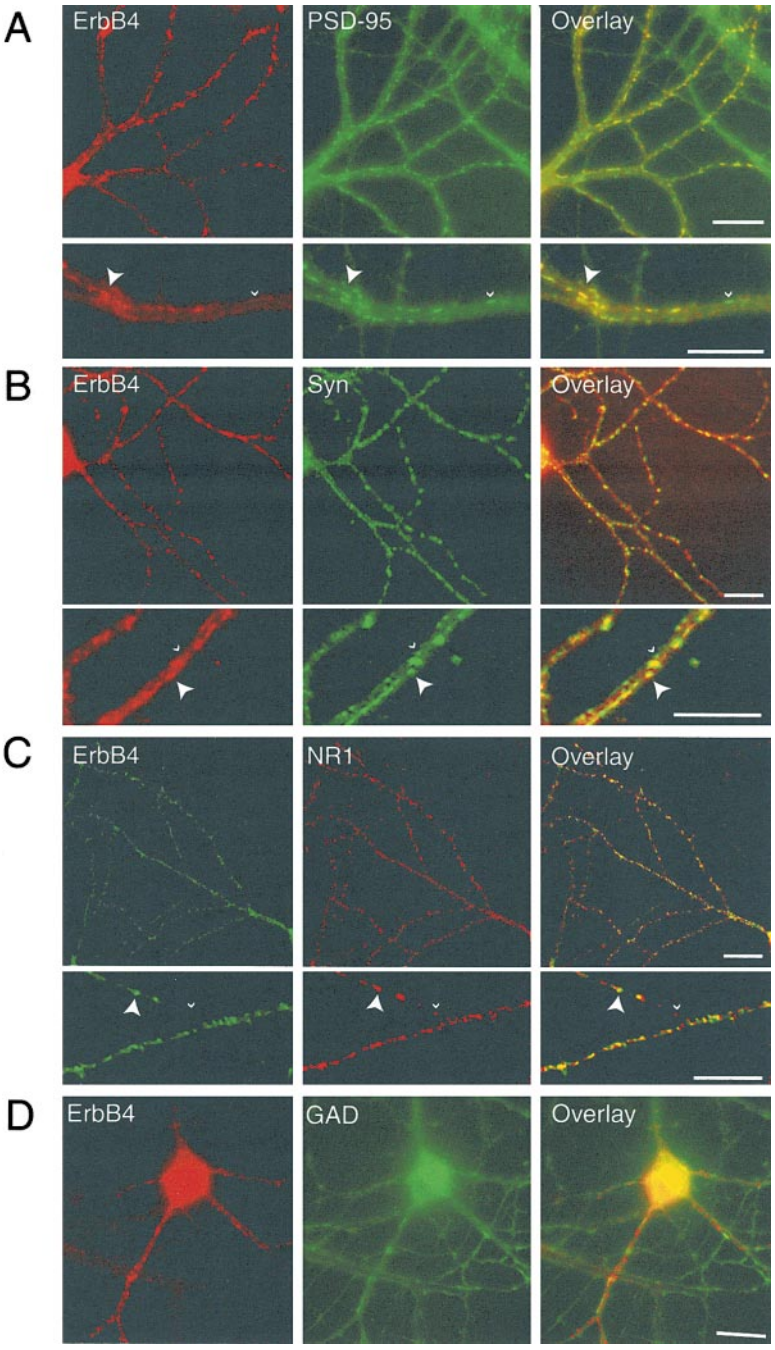

Figure 4. Colocalization between ErbB4 and PSD-95 in Hippocampal Neurons

(A) Hippocampal neurons (14 DIV) were stained with ErbB4 polyclonal and PSD-95 monoclonal antibodies. ErbB4 staining was visualized with a rhodamine-coupled secondary antibody and PSD-95 a FITC-coupled secondary antibody. Labeling of neurons is eliminated by preabsorption of the antibody with its antigen (data not shown). Large arrow indicates colocalization of ErbB4 and PSD-95. Small arrow indicates synapses where PSD-95 but not ErbB4 is present.

(B) Colocalization of ErbB4 with synaptophysin. Hippocampal neurons were stained with ErbB4 polyclonal and synaptophysin (SYN) monoclonal antibodies that were visualized with rhodamine- and FITC-coupled secondary antibodies, respectively. Arrows indicate costaining of ErbB4 and synaptophysin.

(C) Colocalization of ErbB4 with NR1. Hippocampal neurons were stained with ErbB4 monoclonal antibodies and NR1 polyclonal antibodies that were visualized with FITC- and rhodamine-coupled secondary antibodies, respectively. Arrows indicate costaining of ErbB4 and NR1.

(D) Localization of ErbB4 in GAD-positive neurons. Hippocampal neurons were stained with ErbB4 polyclonal and GAD monoclonal antibodies that were visualized with rhodamine- and FITC-coupled secondary antibodies, respectively. GAD-positive neurons were costained with ErbB4. Bar, 10 μm.

costaining of PSD-95, ErbB4 was not present in all synaptophysin-positive synapses (Figure 4B). PSD-95 interacts with various proteins, including NR2, a subunit of the NMDAR (Kornau et al., 1995). To determine whether ErbB4 is enriched at synapses where the NMDAR is enriched, we costained hippocampal neurons with monoclonal antibodies against ErbB4 and polyclonal antibodies against NR1. ErbB4 colocalized with NR1 at synapses in hippocampal neurons (Figure 4C). About 10% of hippocampal neurons in culture stained with antibodies against glutamic acid decarboxylase (GAD), a marker of GABAergic interneurons. Most of the GAD-positive neurons stained positive with ErbB4 antibodies (Figure 4D). In addition, synaptic ErbB4 was also detected in GAD-negative neurons. These results indicate that ErbB4 is expressed not only in pyramidal neurons but also in GABAergic interneurons, in agreement with studies which show that ErbB4 messenger RNA is expressed in areas where GABAergic neurons are clustered (Lai and Lemke, 1991).

Increase in the NRG Signaling by PSD-95

We hypothesized that signaling via ErbB4 may be regulated by PSD-95 to achieve signaling specificity or to ensure correct biological response. To test this hypothesis, we determined whether PSD-95 affects ErbB4 signaling by measuring NRG-induced activation of ErbB4

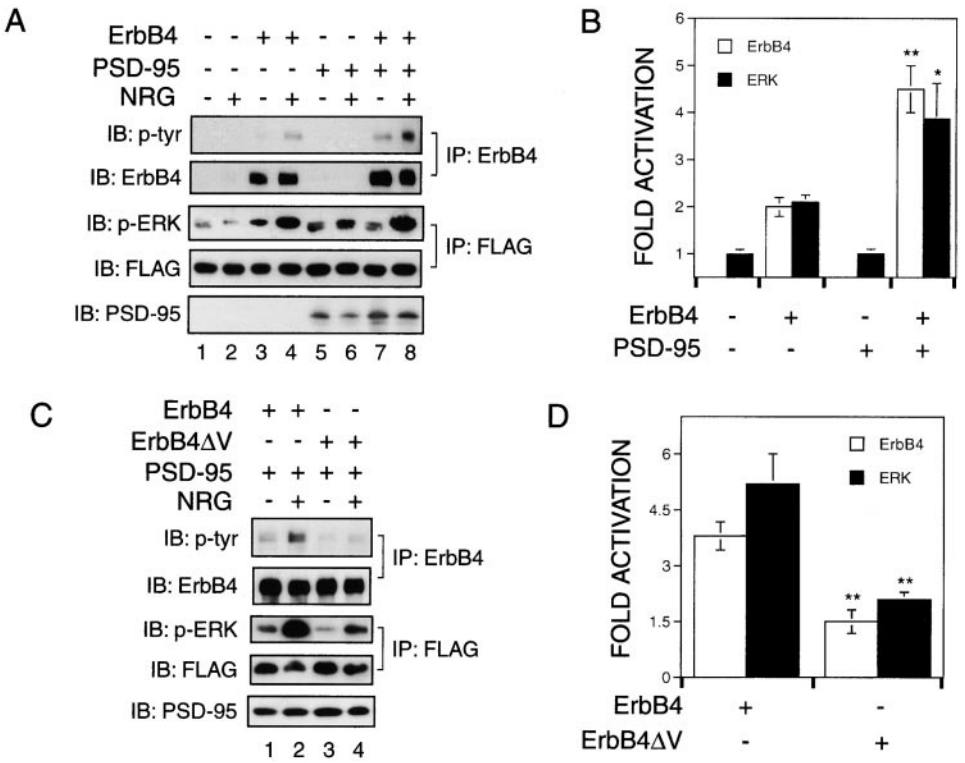

Figure 5. Increase in NRG-Mediated ErbB4 Tyrosine Phosphorylation and ERK Activation by PSD-95

(A) Western blot demonstrating representative effect of PSD-95 on ErbB4 and ERK activation. COS cells were transfected with FLAG-ERK2, without or with ErbB4 and/or PSD-95. Cells were stimulated without (control) or with NRG for 10 min. ErbB4 and FLAG-tagged ERK were immunoprecipitated from the cell lysates and probed with antiphospotyrosine, anti-ErbB4, anti-phospho-ERK antibodies, and anti-ERK antibodies, respectively. Similar results were obtained in more than three independent experiments.

(B) Densitometric analysis of ErbB4 and ERK activation in (A). Autoradiograms were scanned with Personal Densitometer, and the captured image analyzed with the ImageQuant software (Molecular Dynamics). Shown are means ± SD of three or more different samples. Double asterisk, $p < 0.01$, asterisk, $p < 0.05$ in comparison with activation in the absence of PSD-95, Student's t test.

(C) Western blot demonstrating that the effect of PSD-95 on ErbB4 and ERK activation was dependent on the interaction between PSD-95 and ErbB4. COS cells were transfected with FLAG-ERK2, PSD-95, and ErbB4 or ErbB4ΔV. Cells were stimulated without or with NRG for 10 min. ErbB4 and FLAG-tagged ERK were immunoprecipitated from the cell lysates and probed with antiphospotyrosine, anti-ErbB4, anti-phospho-ERK antibodies, and anti-ERK antibodies, respectively. Similar results were obtained in more than three independent experiments.

(D) Densitometric analysis of ErbB4 and ERK activation in (C). Data were analyzed as in (B). Shown are means ± SD of three or more different samples. Double asterisk, $p < 0.01$, in comparison with activation in cells coexpressing ErbB4 and PSD-95, Student's t test.

and ERK, a kinase downstream of ErbB proteins (Si et al., 1996). ErbB4 and an epitope-tagged ERK (FLAG-ERK) were expressed in COS cells. NRG caused an increase in ErbB or ERK activation only in cells transfected with ErbB4 (Figure 5A), consistent with the known expression in COS cells of only ErbB2, which is unable to bind to NRG (Peles et al., 1993). Expression of PSD-95 alone had no consistent effect on the activity of either kinase in COS cells. However, coexpression of ErbB4 with PSD-95 significantly increased NRG-stimulated tyrosine phosphorylation of ErbB4 (from 1.9 ± 0.21–fold, mean ± SEM, to 4.5 ± 0.60–fold over basal, $p < 0.01$) and phospho-ERK (from 2.0 ± 0.05– to 3.8 ± 0.78–fold over basal, $p < 0.05$) (Figures 5A and 5B). These results suggest that PSD-95 may facilitate NRG/ErbB4 signaling. To determine whether the effect of PSD-95 on NRG signaling was dependent on the interaction between PSD-95 and ErbB4, PSD-95 was coexpressed with ErbB4ΔV, a mutant that did not interact with PSD-95 (Figure 1). As shown in Figures 5C and 5D, the increase in ErbB4 kinase activity and ERK activity by NRG was diminished in COS cells coexpressing PSD-95 and ErbB4ΔV, indicating that the PSD-95 regulation of NRG signaling requires the intact C terminus of ErbB4.

To explore further the role of PSD-95 in the regulation of NRG signaling, we examined the effect of suppressing PSD-95 expression on NRG signaling in neurons. Treating cultured cortical neurons with antisense oligonucleotides directed at PSD-95 (5 μM for 10 days) decreased PSD-95 expression to 20% but had no effect on expression of ErbB4 and ERK (Figures 6A and 6B). PSD-95 expression did not change significantly in sham-treated neurons or neurons treated with sense or missense oligonucleotides. In these groups of neurons, NRG stimulated ERK activity by 2.5- to 3-fold. However, NRG-mediated ERK activation was significantly impaired in PSD-95-deficient neurons (Figure 6C). Together these results show that PSD-95 enhances signaling via the NRG-activated ErbB4 receptor.

Ternary Complex between PSD-95 and Two Molecules of ErbB4

Dimerization is an important mechanism to activate receptor tyrosine kinases including ErbBs (Carraway and

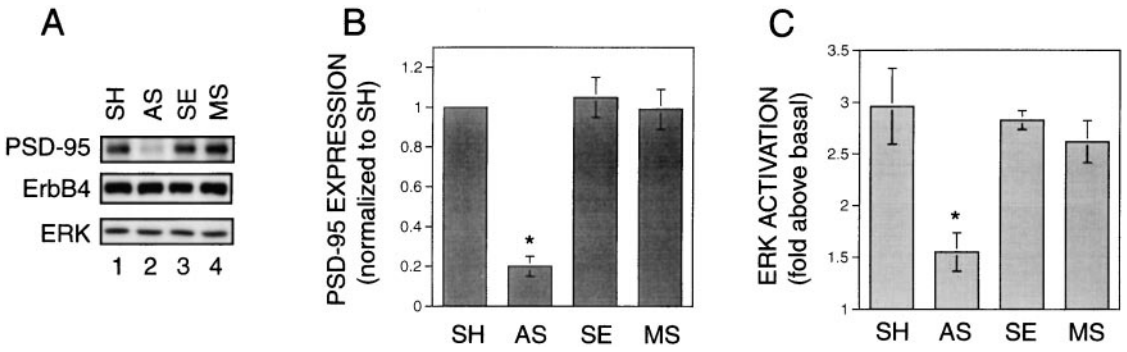

Figure 6. Decrease of NRG-Stimulated ERK in PSD-95-Deficient Neurons

(A) Western blot demonstrating representative effects of sham wash (SH) and PSD-95 antisense (AS), sense (SE), and missense (MS) oligonucleotides on PSD-95 expression in cultured cortical neurons. Neurons were treated for 10 days with 5 μM fully phophorothioated oligonucleotides. Lysates were probed with antibodies against PSD-95, ErbB4, and ERK, respectively.

(B) Densitometric analysis of PSD-95 expression. Data (means ± SD) were from three independent experiments shown in (A). Asterisk, $p < 0.01$ in comparison with SH.

(C) Decreased activation of ERK in PSD-95-deficient neurons. ERK was immunoprecipitated using anti-ERK antibodies and assayed in vitro using myelin basic protein as a substrate in the presence of [γ-^{32}P]ATP as described previously (Si et al., 1996). Asterisk, $p < 0.01$ in comparison with SH.

Cantley, 1994). Because both the first and second PDZ domains of PSD-95 interact with ErbB4, PSD-95 could bind two molecules of ErbB4 at the same time and thus may put receptors in close proximity to facilitate ErbB4 dimerization and signaling. To test this hypothesis, we examined whether PSD-95 forms a ternary complex with two molecules of ErbB4 in cells. The full-length protein and FLAG-tagged C terminus of ErbB4 were cotransfected in COS cells with or without PSD-95. ErbB4 was not detected in immunoprecipitates using anti-FLAG antibodies (Figure 7A), indicating that the two ErbB4 proteins did not interact with each other. In cells cotransfected with PSD-95, however, the full-length ErbB4 copurified with FLAG-tagged ErbB4 C terminus. These results lead to the conclusion that PSD-95 was able to interact with two molecules of ErbB4 in cells to form a ternary complex. ErbB4ΔV, which does not interact with PSD-95, was unable to form the ternary complex, demonstrating the dependence on functional C termini of both ErbB4 proteins and the specificity of anti-FLAG antibodies (Figure 7B). ErbB2 also did not appear to form a ternary complex with ErbB4 and PSD-95 (Figure 7C), suggesting either that PSD-95 was unable to bind to ErbB2 and ErbB4 at the same time or that the weak association between ErbB2 and PSD-95 did not survive immunoprecipitation.

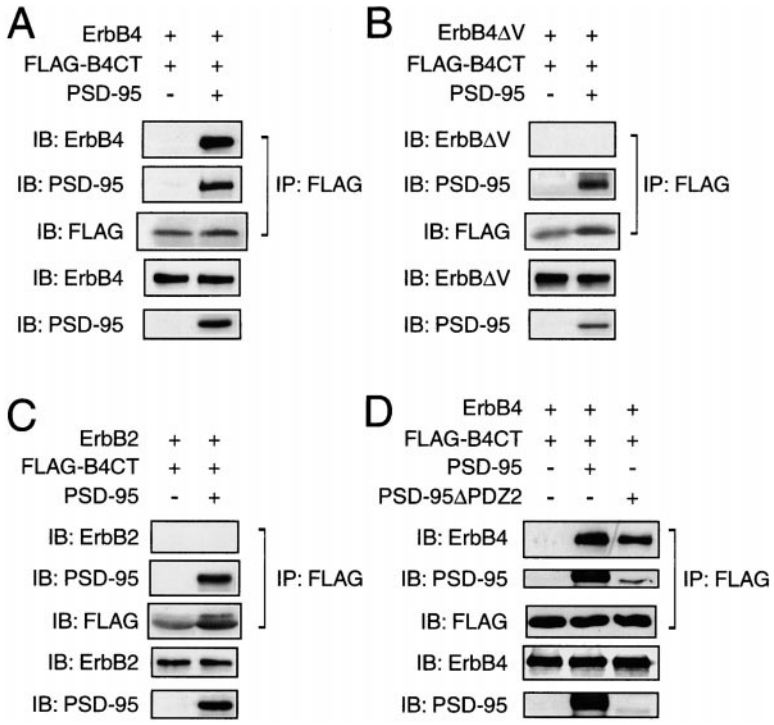

Figure 7. Ternary Complex Formation between ErbB4 and PSD-95

(A) COS cells were transfected with full-length ErbB4 and FLAG-tagged ErbB4 C terminus (amino acids 1143–1308, FLAG-B4CT) without or with PSD-95.

(B) COS cells were transfected with full-length ErbB4 with deletion of the last amino acid residue valine in the C terminus (ErbB4ΔV) and FLAG-B4CT without or with PSD-95.

(C) COS cells were transfected with full-length ErbB2 and FLAG-B4CT without or with PSD-95.

(D) COS cells were transfected with full-length ErbB4 and FLAG-B4CT without or with PSD-95 or PSD-95ΔPDZ2.

FLAG-B4CT was immunoprecipitated with anti-FLAG antibodies and probed with antibodies against ErbB4, PSD-95, FLAG-B4CT, or ErbB2. Cell lysates were also probed directly with antibodies against ErbB4, PSD-95, or ErbB2 to demonstrate inputs. Since the anti-PSD-95 antibody was generated against a recombinant protein containing both the first and second PDZ domains, this antibody interacted weakly with PSD-95ΔPDZ2. A reblot with anti-*myc* antibody indicated abundant expression of *myc*-tagged PSD-95ΔPDZ2 in transfected cells (data not shown).

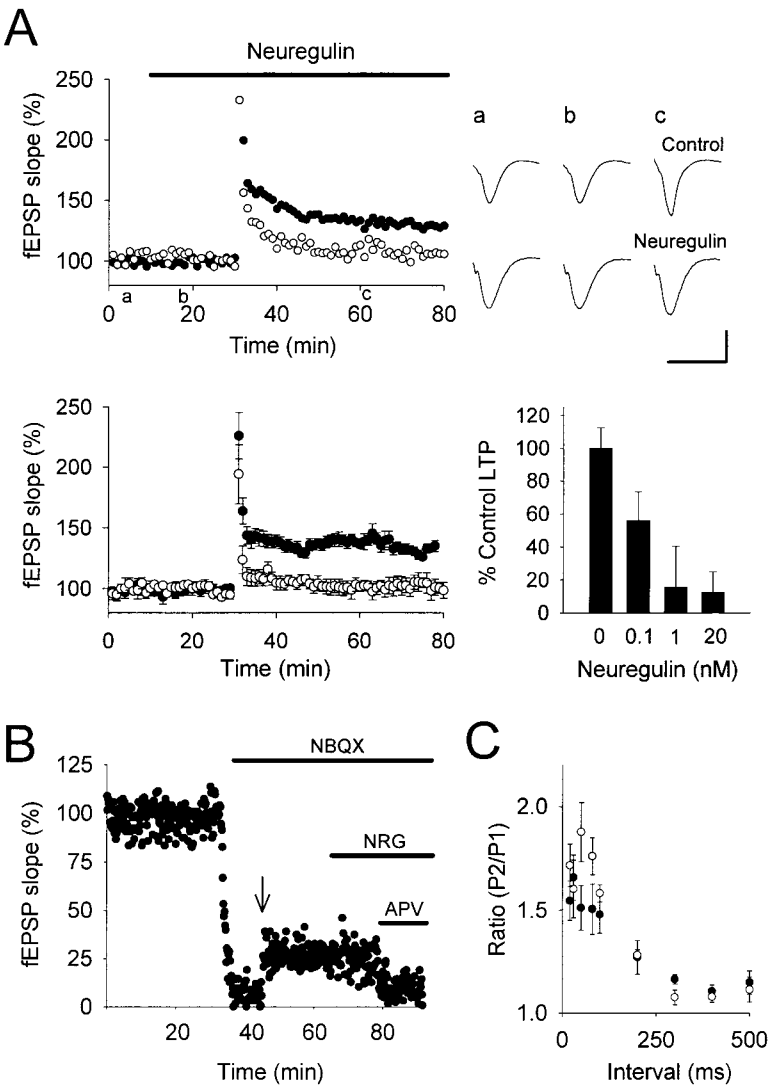

Figure 8. NRG Suppresses Induction of LTP at Schaffer Collateral-CA1 Synapses

(A) Top-field EPSP (fEPSP) slope is plotted every 1 min for a control slice (closed circles) or for a slice treated with NRG (20 nM, open circles) that was applied during the period indicated by the bar. On the right are averaged traces taken before (a), 5 min after starting NRG application (b), and 30 min after tetanus (c). Bottom, normalized fEPSP slope (mean ± SEM) is plotted from control slices (n = 8, closed circles) or slices in which NRG was applied starting 20 min before tetanus (n = 6, open circles). fEPSP slope was normalized with respect to the average slope 5 min before tetanus. Scale bars, 20 ms, 0.5 mV upper, 1 mV lower. Histogram showing LTP as percentage of control level (no NRG) 60 min after tetanus at NRG concentrations of 0.1 (n = 3), 1 (n = 3), and 20 nM.

(B) The plot shows fEPSP slope from a slice in which the ACSF contained bicuculline (2 μM). The AMPA receptor antagonist NBQX (10 μM) was applied as indicated. At the time shown by the arrow, the stimulus intensity was increased. NRG (NRG, 20 nM) was applied as indicated. At the end of the recording, the NMDAR antagonist APV (50 μM) was bath applied as shown to demonstrate that the synaptic response recorded in the presence of NBQX was mediated by NMDARs.

(C) Paired-pulse facilitation of EPSPs before (closed circles) or during (open circles) application of NRG (20nM). At each interstimulus interval, PPF was not statistically significantly different ($p > 0.05$) before versus during NRG. Interstimulus interval is indicated on the x axis. P1, first response; P2, second response.

Recent studies indicate that PSD-95 can form head-to-head multimers via disulfide linkage of its N terminus (Hsueh et al., 1997). Therefore, in addition to the ternary complex of two ErbB4s with PSD-95 due to divalent binding, ErbB4 could bind to multimerized PSD-95s to form clusters as Kv1.4 potassium channels do. Indeed, ErbB4 did form multimers with PSD-95ΔPDZ2, a mutant whose second PDZ domain was deleted (Hsueh et al., 1997) (Figure 7D). These results may suggest that PSD-95 with a single functional PDZ domain is sufficient to form a multimeric complex with ErbB4 due to its head-to-head multimerization.

Suppression of Tetanus-Induced LTP by NRG

Because ErbB4 localizes to excitatory synapses, we questioned whether NRG may affect synaptic transmission or plasticity. We recorded field potentials from the CA1 dendritic region of hippocampal slices and evoked excitatory synaptic responses by stimulating the Schaffer collateral input. We found that bath applying NRG (20 nM) had no effect on basal field EPSP slope (Figure 8A) or on posttetanic potentiation. In contrast, applying NRG suppressed long-term potentiation (LTP) of synaptic transmission: the slope of the field EPSPs was 104% ± 4% (mean ± SEM) of baseline 30 min after tetanus in slices treated with NRG (n = 6) versus 130% ± 4% of baseline in untreated, control slices (n = 8, $p < 0.01$). The inhibitory effect of NRG on LTP induction was concentration dependent with 0.1 nM producing ~50% reduction in the level of potentiation as compared with control (Figure 8A). Induction of LTP is prevented by blocking NMDARs (Malenka and Nicoll, 1999), but NRG had no apparent effect on NMDAR-mediated synaptic responses (n = 3 slices, Figure 8B). Moreover, NRG did not affect paired-pulse facilitation, a measure of presynaptic function (Figure 8C). Thus, NRG depressed the induction of LTP with no effect on basal synaptic transmission, presynaptic facilitation, posttetanic potentiation, or NMDAR function.

Discussion

The present study demonstrates that the NRG receptor ErbB4 is a component of the PSD. PSD-95 interacts with

ErbB4 C terminus and may thus function in regulation of NRG signaling through ErbB4. Indeed, ErbB4 colocalizes with PSD-95 at synapses in hippocampal neurons. Expression of PSD-95 enhances ErbB4 tyrosine kinase activity and NRG-mediated activation of ERK in transfected COS cells. Moreover, inhibition of PSD-95 expression in cultured neurons attenuates NRG-induced ERK activity. These results suggest a role of PSD-95 in regulation of NRG signaling in addition to clustering synaptic components at the PSD. The synaptic localization of ErbB proteins suggested to us that NRG may play an important role in regulation of synaptic transmission. We demonstrated that NRG suppresses tetanus-induced LTP in hippocampal slices.

Synaptic ErbB4 in Adult CNS

ErbB4 is expressed not only in developing CNS but also in adult brain (Plowman et al., 1993b). The ErbB4 mRNA is expressed in a subset of neurons throughout all fields of the hippocampus, dentate gyrus, and neocortex, and in the medial habenula, the reticular nucleus of the thalamus, cerebellar granule cells, and the spinal cord (Lai and Lemke, 1991; Francoeur et al., 1995; Carraway et al., 1997; Meyer et al., 1997). The mRNAs of *nrg-1*, *nrg-2*, and *nrg-3*, but not of *nrg-4*, are highly expressed in the adult brain (Falls et al., 1993; Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Zhang et al., 1997; Yang et al., 1998; Harari et al., 1999). All four NRGs can bind to and thus activate ErbB4 in cultured cells (Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Zhang et al., 1997; Harari et al., 1999). Thus, ErbB4 in hippocampal neurons can be activated by NRG released from adjacent neurons or by an autocrine mechanism. In addition, ErbB4 in tumor cells or heterologous system can be activated by other members of the EGF family, including β-cellulin, heparin binding EGF, epiregulin, and cripto-1 (Beerli and Hynes, 1996; Riese and Stern, 1998; Bianco et al., 1999; Spencer et al., 2000). Whether these factors are expressed in the CNS is yet to be studied.

We demonstrate that ErbB4 is a PSD protein in the adult brain, suggesting that NRG signaling may be confined at synapses and/or that NRG may play a role in the maintenance and/or regulation of synaptic structure and function. ErbB4 ends with the sequence -TVV* that agrees with the consensus motif for binding to the PDZ domains of PSD-95 (Craven and Bredt, 1998). The C terminus (-VPV*) of ErbB2, however, does not conform to this consensus. It is therefore not surprising that ErbB2 has a weak affinity for PSD-95 in yeast two-hybrid assays and barely interacts with PSD-95 in mammalian cells. Interestingly, however, like ErbB4, ErbB2 is enriched in the PSD subcellular fraction. The enrichment of ErbB2 at the PSD may result from heterodimerization with ErbB4 or interaction with a different PDZ protein. ErbB3 but not ErbB4 is expressed in neural crest and Schwann cell precursors during development and subsequently in neurocrest-derived neurons and Schwann cells in adult animals (Meyer et al., 1997). Except for a few cell types such as skeletal muscle and epithelia that express both ErbB4 and ErbB3, the two receptors are distributed in a complementary expression pattern. Consistent with these results, we show that ErbB3 is not present at the PSD.

At the NMJ, all three ErbB proteins are concentrated at the postjunctional membrane (Altiok et al., 1995; Moscoso et al., 1995; Zhu et al., 1995). They play a key role in mediating synapse-specific synthesis of acetylcholine receptors in response to motoneuron-released NRG (Fischbach and Rosen, 1997). The finding that ErbB4 binds to PSD-95 in the PSD may shed light on the mechanisms of ErbB protein aggregation at the NMJ, which only occupies $<0.1\%$ of total muscle fiber surface. Several PDZ domain–containing proteins have been identified that are expressed in skeletal muscles including the syntrophins, a family of cytoplasmic proteins that bind to the carboxyl termini of dystrophin, utrophin, and dystrobrevin (Peters et al., 1994; Lumeng et al., 1999). In muscle, α1- and β1-syntrophin localize to the sarclemma and NMJ, whereas β2-syntrophin is found only at the NMJ. Interestingly, ErbB4 interacts with the PDZ domain of α1-syntrophin, but not β1- or β2-syntrophins. Determination of whether PDZ domain–containing proteins in fact aggregate ErbB proteins at the NMJ requires further studies.

Regulation of NRG Signaling by PSD-95

Most PSD-95 interacting proteins appear to be constituents of the PSD since they colocalize with PSD-95 in cultured cells and coimmunoprecipitate in biochemical studies. Some of these proteins have no apparent enzymatic activity, such as CRIPT (Niethammer et al., 1998), GKAP/SAPAP (Kim et al., 1997; Takeuchi et al., 1997), and BEGAIN (Deguchi et al., 1998). They are believed to play a role in the formation or maintenance of synapses. Remarkably, PSD-95 also binds to proteins with important signaling functions, including NMDARs (Kornau et al., 1995), K^+ channels (Kim et al., 1995), SynGAP (Chen et al., 1998; Kim et al., 1998a), nNOS (Brenman et al., 1996), and PMCA4b (Kim et al., 1998b). Localization of these proteins is believed to facilitate neurotransmission and/or restricted signaling at synapses. However, there is no evidence that the activity of any of these signaling proteins can be regulated by PSD-95, either directly or indirectly. Coexpression of PSD-95 increased NRG-dependent ERK activation through ErbB4. Conversely, suppression of PSD-95 expression with antisense oligonucleotides decreased NRG-activated ERK. These results demonstrated that ErbB signaling is regulated by PSD-95.

Ligand binding to receptor tyrosine kinases causes dimerization of the receptors and activation of kinase activity, which is followed by intermolecular transphosphorylation and subsequent phosphotyrosine-mediated interaction with adapter proteins. We demonstrate that ErbB4, in addition, can interact with a protein(s) in a phosphotyrosine-independent manner. Such interaction may play an important role in the regulation of NRG signaling. Our results suggest that PSD-95 may bring ErbB4 receptors in proximity for dimerization and thus enhance NRG signaling. Alternatively, PSD-95, by interacting with the ErbB4 protein tyrosine kinase, may become tyrosine phosphorylated and function as an adapter protein to transduce signals to downstream signaling components. Recent data have demonstrated that coexpression of ErbB4 does not lead to tyrosine phoshorylation of PSD-95 (S. W. and L. M., unpublished

data). On the contrary, coexpression of PSD-95 increased the phosphotyrosine content of the NRG-stimulated ErbB4. These results demonstrate that the site of PSD-95 action is at the level of ErbB4, supporting the hypothesis that PSD-95 regulates NRG signaling by facilitating ErbB4 dimerization. ErbB2 had a weak affinity for the PDZ domains of PSD-95 and was unable to form a ternary complex with ErbB4 and PSD-95. Therefore, the PSD-95 regulation of NRG signaling may be limited to that through ErbB4 homodimers. PSD-95 is a scaffold protein, interacting with various signaling proteins, including SynGAP (Chen et al., 1998; Kim et al., 1998a), nNOS (Brenman et al., 1996), and PMCA4b (Kim et al., 1998b). It is possible that NRG signaling may also be regulated by these proteins. Of particular interest is SynGAP, a Ras-GTPase activating protein at excitatory synapses, which stimulates the GTPase activity of Ras and thus may negatively regulate Ras activity.

Synaptic Plasticity and NRG

In the CA1 region of the hippocampus, a brief train of high-frequency stimulation produces the lasting enhancement of synaptic transmission known as LTP. Stimulation of the NMDA class of glutamate receptors is a key step in the mechanism of LTP (Salter, 1998; Malenka and Nicoll, 1999). Our findings that NRG suppressed induction of LTP in CA1 but had no effect on paired-pulse facilitation together with the localization of ErbB4 in the PSDs suggest that NRG acts via a postsynaptic mechanism. The activity of NMDARs is subject to modulation by tyrosine phosphorylation (Wang and Salter, 1994; Yu et al., 1997), but the suppression of LTP could not be attributed to an effect on the amplitude of NMDA currents because NRG did not alter NMDA receptor–mediated synaptic responses. Thus, NRG/ErbB4 signaling may interrupt LTP induction at a step beyond NMDAR stimulation. ErbB4 has an extended C-terminal region that contains numerous tyrosine residues. Upon phosphorylation, these tyrosine residues bind to adapter proteins and activate a diversity of signaling pathways including ERK, JNK, and PI3 kinase (Burden and Yarden, 1997). One of these kinase signaling cascades may have a role in the suppression of synaptic plasticity by NRG.

NRG-2 and NRG-3 are expressed in adult hippocampus (Busfield et al., 1997; Carraway et al., 1997; Zhang et al., 1997). At the cellular level, *nrg* mRNAs are detected in granule cells of the hippocampus and the dentate gyrus (Busfield et al., 1997; Carraway et al., 1997). There are at least 14 different splice variants of the *nrg-1* mRNAs, which encode proteins containing an α- or β-type epidermal growth factor (EGF) domain and either an immunoglobulin- (Ig-)like domain or a cysteine-rich domain (CRD) at the N terminus (Fischbach and Rosen, 1997; Wolpowitz et al., 2000). The Ig-containing but not CRD-containing NRG-1 is expressed in adult hippocampus (Corfas et al., 1995; Carraway et al., 1997). All NRG functions known to date can be duplicated by the EGF domain (Fischbach and Rosen, 1997). The NRG used in this study, $rHRG_{\beta177–244}$, is a recombinant polypeptide containing the entire EGF domain (amino acids 177–244) of the β-type NRG-1, a potent isoform (Holmes et al., 1992). $rHRG_{\beta177–244}$ binds to ErbB3 and ErbB4 and thus induces tyrosine phosphorylation of ErbB2, ErbB3, and ErbB4, but not of the EGF receptor (Jones et al., 1998; Tanowitz et al., 1999; this study; J. Si and L.M., unpublished data). Thus, the effect of $rHRG_{\beta177–244}$ on LTP is believed to be mediated via activation of the NRG signaling pathway. Exactly which NRGs or NRG isoforms are involved in this event requires further studies.

Experimental Procedures

Yeast Two-Hybrid Studies

The ErbB4 carboxyl terminus (amino acid residues 1216–1308) (Plowman et al., 1993b) was generated by PCR, subcloned into pGBT9 (Clontech), and used as a bait to screen a pool of mouse brain and muscle (oligo-dT-primed) cDNA libraries. Two hundred eleven were positive on plates lacking leucine, tryptophan, and histidine with 1 mM 3-aminotriazole, nine of which were further confirmed by filter assay for β-galactosidase activity: one encoding PSD-95, two encoding SAP102, and the rest encoding novel proteins. ErbB4 carboxy-terminal deletion mutants and ErbB2 (1215–1260) and ErbB3 (1245–1339) carboxyl termini were generated by PCR and subcloned in pGBT9, pGAD424 (Clontech), or pPC97 (Dong et al., 1997). PDZ domains of PSD-95 [PDZ1-3 (62–393), PDZ1 (62–151), PDZ2 (158–246), PDZ3 (313–393), PDZ1-2 (62–246), and PDZ2-3 (158–393)], SAP102 (119–849), nNOS (1–139), PTP-BAS2 (1349–1433), α1-syntrophin (88–166), β1-syntrophin (113–191), β2-syntrophin (116–194), and GRIP (30–331) were generated by PCR and subcloned in pGAD424, pACT2 (Clontech), or pPC86 (Dong et al., 1997). The yeast vectors are transformed into HF7c or SFY526. Interactions were characterized by growth without leucine, tryptophan, and histidine and by a filter assay for β-galactosidase activity. The affinity of PSD-95 with ErbB4 and ErbB2 was also characterized in liquid β-galactosidase assays as described previously (Guarente and Mason, 1983).

Subcellular Fractionation

Adult rat brains were homogenized with a glass-Teflon homogenizer. Subcellular fractions were prepared as described previously (Blackstone et al., 1992).

Cell Culture, Transfection, and Treatment

Hippocampal neuronal cultures were prepared from 18-day-old embryonic rats following the procedures as described (Banker and Cowan, 1977). HEK293 cells and COS cells were transfected using the standard calcium phosphate technique. NRG used was a recombinant peptide of HRGβ1 residues 177–244 ($rHRG_{\beta1177–244}$) (Holmes et al., 1992). Antisense, sense, and missense oligonucleotides have been described previously (Sattler et al., 1999).

Immunoprecipitation and Immunoblotting

The rat brain P2 fraction was solubilized in 1% sodium deoxycholate, 50 mM Tris–HCl (pH 9.0) at 36°C for 30 min. After the addition of Triton X-100 to a final concentration of 0.1%, the preparation was incubated at 4°C overnight and then centrifuged at 37,000 $\times$ g for 30 min. The solubilized P2 fraction or lysates of transfected cells (300–500 μg of protein) were incubated with specific antibodies immobilized on protein A–agarose beads as described previously (Won et al., 1999). Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotting. Antibodies used were from Upstate Technology (PSD-95, NR1, and GluR1), Santa Cruz (ErbB2, ErbB3, ErbB4, and c-*myc*), Transduction Labs (antiphosphotyrosine antibodies), and Sigma (anti-FLAG antibodies).

Immunocytochemistry

Low-density hippocampal neurons, after 2 weeks in culture, were fixed with 4% paraformaldehyde for 20 min, blocked with 4% donkey serum or BSA, and incubated with antibodies against ErbB4 (monoclonal, NeoMarkers; polyclonal, Santa Cruz), PSD-95 (Upstate Technology), synaptophysin (Chemicom), and/or GAD (Chemicon). Double-label immunostaining was done with appropriate fluorochrome-conjugated secondary antibodies. Fluorescent images of cells

were captured on a Sony CCD camera mounted on a Nikon E600 microscope using Photoshop imaging software.

Eletrophysiology

Methods for preparing slices and extracellular field recording are described in detail elsewhere (Lu et al., 1998). Briefly, hippocampal slices were prepared from 4- to 6-week-old male Sprague–Dawley rats and were placed in a holding chamber for at least 1 hr prior to recording. A single slice was transferred to a recording chamber and superfused with artificial cerebral spinal fluid (ACSF, 2 ml/min) composed of (in mM) 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose, saturated with 95% O_2/5% CO_2 at 30°C ± 2°C. The ACSF was supplemented as required with NRG that was stored as 10,000× single-use aliquots in water. Synaptic responses were evoked with bipolar tungsten electrodes located about 50 μm from the cell body layer in CA1. Test stimuli were evoked at 0.1 Hz with the stimulus intensity set to 25% of that which produced maximum synaptic responses. Tetanic stimulation consisted of two trains of 100 Hz stimuli lasting 500 ms, at an intertrain interval of 10 s. Field potential recordings were made with glass micropipettes filled with ACSF placed in the stratum radiatum 60–80 μm from the cell body layer. Field EPSP slope was calculated as the slope of the rising phase between 10% and 60% of the peak response. Synaptic responses were stable by 30 min following tetanus and persisted at this level for >1.5 hr. For recording NMDA receptor–mediated field responses, ACSF contained 1 mM $MgCl_2$ and was supplemented with bicuculline (5 μM) and NBQX (10 μM).

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